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Strategies for increasing aroma production from castor oil by *Yarrowia lipolytica*

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Work developed under supervision of
Supervisor: Professora Doutora Isabel Belo

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STATEMENT

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"Whatever you do will be insignificant, but it is very important that you do it."

Mahatma Gandhi

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Finda esta longa jornada, não posso deixar de agradecer a todos os que a tornaram mais fácil e que, de uma forma ou de outra, deixaram um bocadinho de si (palavras, acções, sorrisos, brincadeiras) na bagagem que hoje trago comigo...

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ABSTRACT

The aromatic compounds produced by biotechnological processes are increasingly accepted by consumers because they are considered as "natural" compounds. In addition, they are of great interest due to the high yields obtained comparatively to chemical processes. γ-Decalactone is an aromatic compound of industrial interest, resulting from the peroxisomal β-oxidation of ricinoleic acid. This fatty acid, the major constituent of castor oil, is the precursor most commonly used in the biotechnological production of this aroma. Although there are many works described in the literature about aroma production, several factors remain to be fully understood and optimized in order to improve γ-decalactone production.

Thus, this work initially aimed to study the influence of lipase in castor oil hydrolysis and the consequent impact in the aroma production. Lipozyme TL IM® revealed to be an efficient lipase to hydrolyze castor oil (95.4 % of hydrolysis in 48 h). In spite of the higher aroma concentration obtained without lipase, the process was faster when Lipozyme TL IM® was involved, resulting in similar productivities.

One of the limitations in the development of an industrial process for γ-decalactone production is the toxicity of the substrate and the lactone. The immobilization by adsorption of *Y. lipolytica* W29 in methyl polymetacrilate and DupUm® was studied in flasks batch cultures. After selecting the best conditions for cell immobilization, free and immobilized cells were used in the biotransformation of ricinoleic acid into γ-decalactone. The results demonstrated an improvement in γ-decalactone concentration with adsorbed *Y. lipolytica* cells on DupUM® since a greater amount of γ-decalactone was accumulated in the medium compared to free cells. In this case the use of the extracellular lipase Lipozyme TL IM® in the biotransformation medium was crucial to allow castor oil hydrolysis, without it the substrate access to the cells would be impossible. Furthermore, immobilized cells hold a stable γ-decalactone production after being stored for 30 days at 4 °C. Also after reuse in three consecutive biotransformations, γ-decalactone concentration was ca. 80 % of that in the first cycle, indicating that immobilized cells could be reused for at least three cycles.

The production of γ-decalactone in batch cultures of *Y. lipolytica* W29 free cells was studied at bioreactor level and the preformance in stirred tank (STR) and airlift bioreactors was compared. The oxygen mass transfer was characterized and the positive influence of k_La on γ-decalactone productivity was demonstrated for both bioreactors. A 2-fold increase in γ-decalactone concentration was achieved in the airlift when compared to STR; but, in this last bioreactor the production rate was higher. Morphological characterization of the yeast cells by image analysis showed that pneumatic agitation causes less impact in the cells morphology than mechanical agitation. A predominance of loose cells and quite irregular structures was observed in the STR. So, the airlift bioreactor presents potential interest for larger scale production, with important cost savings, due to the reduction of power input consumption.

Finally, the performance of *Y. lipolytica* strains with modifications in the lipid metabolism at the β-oxidation pathway (acyl-CoA oxidases) and the triglyceride hydrolysis (*LIP2* overexpression) using castor oil as substrate was monitored in the STR bioreactor. Depending on genotype, degradation of γ-decalactone was prevented. Also, a faster initial rate of aroma production was obtained with strain overexpressing *LIP2* due to the fast hydrolysis of castor oil and release of ricinoleic acid. Step-wise fed-batch cultures improved γ-decalactone concentration only for MTLY40-2P strain, for which a 1.7-fold increase in γ-decalactone final concentration was achieved.

The present work brings new insights on the biotechnological production of γ-decalactone contributing with some different strategies for increasing aroma production leading to a greater γ-decalactone concentration.

RESUMO

Os compostos aromáticos produzidos por via biotecnológica são cada vez mais aceites pelos consumidores, uma vez que são considerados "naturais", sendo esta uma mais valia perante as atuais preferencias do mercado. Além disso, os rendimentos obtidos por esta via são superiores aos obtidos na produção por síntese química. A γ–decalactona é um composto aromático de elevado interesse industrial que resulta da β-oxidação peroxissomal do ácido ricinoleico. Este ácido gordo, principal constituinte do óleo de rícino, é o precursor mais utilizado para a produção biotecnológica deste aroma. Embora existam muitos trabalhos descritos na literatura neste tópico, são vários os fatores cujo efeito ainda permanace por compreender e, consequentemente optimizar podendo permitir melhorar a produção da γ-decalactona.

Assim, este trabalho teve como objetivo inicial estudar a influência da lípase na hidrólise do óleo de rícino e consequente impacto na produção do aroma. Foi utilizada a enzima Lipozyme TL IM® que foi eficiente na hidrólise do óleo de rícino (95.4 % de hidrólise em 48 h). Apesar dos resultados obtidos demonstrarem que a produção de aroma mais elevada é obtida na ausência de lípase, o processo foi mais rápido quando a Lipozyme TL IM® esteve envolvida, resultando em produtividades idênticas.

Uma das limitações para o desenvolvimento de um processo industrial para a produção de γ-decalactona é a toxicidade do substrato e da própria lactona. A imobilização de *Y. lipolytica* W29 por adsorção em polimetilmetacrilato e DupUm® foi estudada, em modo batch em matraz. Depois de selecionadas as melhores condições de imobilização, foi comparada a produção de γ-decalactona com células livres e imobilizadas. Os resultados obtidos demonstraram que a melhor abordagem para aumentar a concentração de γ-decalactona é a adsorção de células em DupUm® uma vez que, nestas condições, a acumulação de aroma obtida foi semelhante às células livres. Neste caso, a utilização da lípase extracelular Lipozyme TL IM® no meio de biotransformação foi crucial para a hidrólise do óleo de rícino, sem a qual o acesso do substrato às células seria impossível. Além disso, as células imobilizadas podem ser armazenadas até 30 dias a 4 °C mantendo a capacidade de produção de γ-decalactona. Após reutilização das células em três biotransformações consecutivas, a concentração de

γ-decalactona foi de aproximadamente 80 % do valor obtido no primeiro ciclo, indicando que as células imobilizadas podem ser reutilizadas, pelo menos, durante três ciclos.

A produção de aroma com células livres de *Y. lipolytica* W29 em modo batch foi testada em dois biorreatores, tanque agitado (STR) e airlift. A transferencia de oxigénio foi caracterizada em ambos os reatores assim como a influência positiva do k_La na produção da γ-decalactona. A concentração de γ-decalactona duplicou no bioreactor airlift em comparação com o STR, no entanto produtividades mais elevadas são obtidas para o STR. A caracterização morfológica das células por análise da imagem mostrou que a agitação pneumática causa menos impacto na morfologia das células do que a agitação mecânica, sendo que no STR foram observadas, predominantemente, estruturas bastante irregulares. Assim, o biorreator airlift apresenta elevado interesse para a produção de aroma em grande escala uma vez que permite reduzir os custos de operação devido à redução do consumo de energia.

Por fim, foi monitorizado o desempenho de estirpes mutantes de *Y. lipolytica* que possuem deleções génicas no metabolismo dos lípidos na via de β-oxidação (acil-CoA oxidases) e na hidrólise dos triglicéridos (subexpressão do gene *Lip2*), utilizando óleo de rícino como substrato. Dependendo do génótipo, a degradação da γ-decalactona foi impedida. Além disso, uma maior velocidade de produção do aroma foi observada para a estirpe que subexpressa o gene *Lip2*, uma vez que houve uma hidrólise mais rápida do óleo de rícino e consequente libertação do ácido ricinoleico. O modo de operação semi-contínuo com alimentação intermitente permitiu aumentar a produção de γ-decalactona apenas para a estirpe MTLY40-2P, para a qual se observou um aumento de 1.7 vezes na concentração final de γ-decalactona.

Assim, o presente trabalho apresenta novas prespetivas sobre a produção biotecnológica da γ-decalactona, contribuindo com diferentes estratégias que conduziram a um aumento na produção de aroma e permitiram obter uma elevada concentração de γ-decalactona (7 g L^{-1}).

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

a	Gas-liquid interfacial area (m^{-1})
A	Absorbance at 600 nm at the beginning of the MATH test
A_0	Absorbance at 600 nm at the end of the MATH test
$A_{\gamma\text{-decalactone}}$	Area of γ-decalactone
$A_{3\text{-OH-}\gamma\text{-decalactone}}$	Area of 3-hydroxy-γ-decalactone
Aox_i	Acyl-CoA oxidase
C_i	Long chain carbon
CA to E	*Yarrowia lipolytica* chromosome A to E
CO	Castor oil
Comp	Compactness
C-PMMA	Methyl polymethacrylate cylinders
CTAB	Cetyltrimethylammonium bromide
Deq	Equivalent Diameter
DO	Dissolved oxygen
D_i	Impeller diameter (m)
DNS	3,5-dinitrosalicylic acid
er_{obj}	Erosions needed to delete an object
FDA	Food and Drug Administration
FAD	Flavin adenine dinucleotide
$FADH^2$	Reduced flavin adenine dinucleotide
FAME	Fatty acid methyl esther
F_{Cal}	Calibration factor (μm $pixel^{-1}$)

F_g	Volumetric gas flow rate ($m^3\ s^{-1}$)
GC	Gas chromatography
GRAS	Generally Regarded As Safe
H_2O_2	Hydrogen peroxide
K	Response coefficient
k_La	Volumetric mass transfer coefficient (h^{-1})
K_T	Constant dependent on the impeller used
M	Molarity (mol L^{-1})
MATH	Microbial adhesion to hydrocarbons
MM	Molar mass (g mol^{-1})
NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide
N	Agitation rate (s^{-1})
N_P	Power number
N_{Re}	Reynolds number
O	Dissolved oxygen concentration within the reactor (mg L^{-1})
O_2	Oxygen
OD	Optical density (nm)
O_0	Dissolved oxygen concentration when aeration is resumed (mg L^{-1})
O_i	Dissolved oxygen concentration in the benning of biotransformation (mg L^{-1})
O^S	Oxygen saturation concentration (mg L^{-1})
OTR	Oxygen transfer rate (g L^{-1} h^{-1})
OUR	Oxygen uptake rate (g L^{-1} h^{-1})
P_g	Power input of the aerated bioreactor (W)
$P_{g'}$	Power input to the non-aerated system (W)
POX_i	Genes codifying acyl-CoA oxidase

Q_g	Gas flow-rate (L h^{-1})
q_{O2}	Specific oxygen uptake rate (mg $g^{-1}h^{-1}$)
QIA	Quantitative image analysis
R^2	Correlation coefficient
RGB	Red Green Blue
Robus	Robustness
SCoA	Acyl Coenzyme A
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscope
STR	Stirred-tank reactor
SV	Saponification value (mg g^{-1})
t	Time (h)
t_0	Time when the aeration is resumed (h)
U_g	Surface gas liquid velocity (m s^{-1})
U_L	Liquid velocity (m s^{-1})
V	Bioreactor working volume (m^3)
VB	KOH volume dispended in the blank titration (mL)
VS	Titrated sample volume (mL)
vvm	Volume of air per volume of reactor per minute
YPD	Yeast extract, peptone and dextrose medium
YPDA	Yeast extract, peptone, dextrose and agar medium
YNB	Yeast Nitrogen Base
WT	Wild type strain

Subscripts:

f	Final conditions
i	Conditions
0	Initial conditions
O	Oxygen

Greek letters:

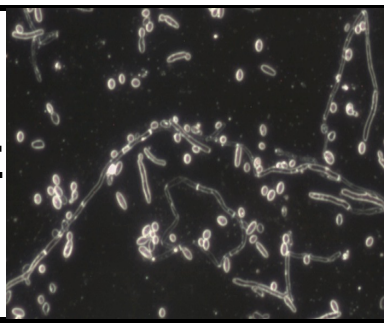
α	Numerical constant
β	Numerical constant
δ	Numerical constant
ζ	Numerical constant
γ	Interfacial tension (mN m^{-1})
γ	Numerical constant
ρ	Liquid density (kg m^{-3})
υ_s	Superficial gas velocity (m s^{-1})

Notes:

In general, the International System of Units (SI) was used in this work. Sometimes multiples and sub-multiples of the SI units were also used, as well as other non-SI units but allowed by SI, such as the use of liter to express volume.

Some units not recognized by the SI were also used to express some variables, such as the volume percent (% v/v) to denote the composition of some solutions, the revolutions per minute (rpm) to indicate the agitation rates and the volume of air per volume of reactor per minute (vvm) to designate the aeration rates, due to the usual use in fermentation technology area.

RESEARCH AIM AND THESIS OUTLINE



This chapter introduces the background information about the theme of the work, as well as its objectives. The outline of the thesis and its output is also presented.

1.1 RESEARCH AIM

The request for new flavourings increases every year. Consumer perception that everything natural is better is causing an increase demand for natural flavour additives and biotechnological routes may be, if they exclude any chemical steps, a way to get natural products.

Lactones are widely used in flavouring industry. They have been found in the aromas of more than 120 foodstuffs (foods and beverages) and have been observed to be associated with aromas described as fruity, coconut-like, buttery, sweet or nutlike (Maga, 1976). γ-Decalactone, which has a characteristic fruity odor, was first reported by Tahara et al. (1973) to be the major volatile constituent present in cultures of *Sporobolomyces odorus*. This fragrant compound has a peach aroma and can be detected in water in concentrations as low as 0.088 ppm (Siek et al., 1971). It is considered a GRAS food additive by the US FDA (Arctander, 1969).

The pathway to γ-decalactone production was discovered by Okui et al. (1963) and is currently the compound with the highest volume of production via a biotechnology process (Gatfield, 1988). Since Okui's publication (Okui et al., 1963), other groups have been exploring this topic, as is the case of Endrizzi-Joran et al. (1993) who discover that β-oxidation pathway was involved in the biotransformation of γ-decalactone, a topic further explored by several authors (Gatfield et al., 1993; Haffner and Tressl, 1996; Spinnler et al., 1996). Feron et al. (1996b, 1997) focused on the toxicity levels of this lactone, while Endrizzi-Joran (1994) has studied in detail the degradation of γ-decalactone. In 2000, Waché and co-works characterized the involvement of acyl-CoA enzymes in the conversion of methyl ricinoleate into γ–decalactone by *Yarrowia lipolytica* with the goal of increasing its production. More recently, scientists have been concentrating on gene function that encodes acyl-CoA oxidase isozymes and selection of over-producing mutants (Groguenin et al., 2004; Guo et al., 2011; Guo et al., 2012). Also, studies concerning the lipid metabolism that lead to lactone production (Waché et al., 2003; García, 2008), the interactions of cells-substrate (Aguedo, 2002a), the importance of oxygen mass transfer in overall process (Aguedo et al., 2005a; Gomes et al., 2007), and the impact of experimental conditions in aroma production (Gomes et al., 2010; Gomes et al., 2012) have been described by several authors. All these studies have improved the knowledge around the mechanisms involved in aroma production. Despite these efforts, the lactone production did not increase substantially. So it is important to understand the mechanisms that regulate this process and find

some possible alternative strategies to increase aroma production.

The main objective of this thesis is to improve the γ-decalactone production in a biphasic system containing castor oil as ricinoleic acid source, by the yeast *Y. lipolytica.* Since the process revealed to be very slow and with low productivities (probably due to an inefficient hydrolysis of the oil), it has been studied the influence of exogenous lipase in castor oil hydrolysis and the consequent impact in the aroma production. One of the limitations in the development of an industrial process for γ-decalactone production is the toxicity of the substrate and the lactone itself (Osumi et al., 1975; Smucker and Cooney, 1981; Kionka and Kunau, 1985). Therefore, an alternative production technique should be considered to protect the cells from the toxicity of ricinoleic acid and thus improve the production of γ-decalactone. The immobilization of *Y. lipolytica* cells was studied and γ-decalactone production was compared with freely suspended cells. Immobilized cells exhibit tolerance to toxic compounds and enhanced the fermentation productivity (Holcberg and Margalith, 1981). Also, several other aspects were studied: substrate concentration, dissolved oxygen concentration, different fermentation strategies: batch and step-wise fed-batch. The production of γ-decalactone was assayed in a STR and compared with an airlift bioreactor. Finally, the characterization of γ-decalactone production by different genotypes *Y. lipolytica* strains and their response to increased substrate concentration was performed.

1.2 OUTLINE OF THE THESIS

The demand of consumers for aromatic compounds produced by natural means has led to a decrease of natural resources used for this purpose, being of great importance the development of biotechnological processes that meet consumer needs. However, in the available literature the production of interest compounds is low, so it is necessary to develop strategies to increase the process productivity. Given this, the main goals of this thesis were to understand the biotechnological pathway for γ-decalactone production and to increase aroma production. Hence, the thesis was structured in eight chapters:

The present section (CHAPTER 1) introduces the background information about the biotechnological production of γ-decalactone and the motivations on finding new strategies to increase the process productivity.

A review on the state of art of the biotechnological production of γ-decalactone, as well as the yeast used for its production: *Y. lipolytica*, cell immobilization and biotransformation in biphasic systems is presented on CHAPTER 2.

CHAPTER 3 describes the general materials and methods used.

In CHAPTER 4, the influence of lipase in castor oil hydrolysis and the consequent impact in the aroma production was studied. On the other hand, the study of different supports and culture conditions that are suitable for *Y. lipolytica* W29 immobilization was also presented. This strategy aims to protect the cells from the toxicity of lactone and substrate and increase the aroma production.

CHAPTER 5 presents information about the different strategies to improve γ-decalactone production by *Y. lipolytica* W29 free cells, focusing on the influence of oxygen transfer rate on γ–decalactone production in stirred tank and airlift bioreactors, and comparing the aroma production in both systems. The influence of agitation type according with each bioreactor in cell morphology was also investigated.

CHAPTER 6 contains the results obtained combining the γ-decalactone production by different genotypes *Y. lipolytica* strains and their response to increased substrate concentration.

CHAPTER 7 presents the overall conclusions and prespectives for future work.

CHAPTER 8 gathers all the references used in the elaboration of this work.

1.3 OUTPUTS OF THIS THESIS

According to the 2nd paragraph of the article 8 of the Portuguese Decree-Law no. 388/70, the scientific outputs of this thesis are listed in references 1-7. Moreover, some conferences were used as means of learning new methodologies and of assessing the acceptance of the scientific community to the new "knowledge" developed throughout this thesis. The works presented in these events are enumerated bellow.

REFERENCES

1) **Braga, A**.; Gomes, N.; Belo, I. (2012) "Lipase Induction in *Yarrowia lipolytica* for Castor Oil Hydrolysis and Its Effect on γ-decalactone Production". J. Am. Oil Chem. Soc. 89(6):1041-1047.

2) **Braga, A**.; Belo, I. (2013) "Immobilization of *Yarrowia lipolytica* for aroma production from castor oil." Appl. Biochem. Biotech. 169(7):2202-11.

3) **Braga, A**.; Gomes, N.; Belo, I. (2013) Impact of lipase-mediated hydrolysis of castor oil on γ-decalactone production by *Yarrowia* lipolytica. J. Am. Oil Chem. Soc. 90(8): 1131-1137.

4) **Braga, A**.; Belo, I. (2014) Production of γ-decalactone by *Yarrowia lipolytica*: insights into experimental conditions and operation mode optimization. J. Chem. Technol. Biot. DOI: 10.1002/jctb.4349

5) **Braga, A**.; Mesquita, D.P.; Amaral, A.L.; Ferreira, E.C.; Belo, I. (2014) Aroma production by *Yarrowia lipolytica* in airlift and STR bioreactors: differences in yeast metabolism and morphology. Biochem. Eng. J. (submitted).

6) **Braga, A**.; Crutz-Le Coq, A.M.; Dulermo, R.; Nicaud, J.M.; Belo, I. (2014) Effect of *POX* genotype and *Lip2p* overexpression on lactone production and reconsumption during biotransformation of castor oil by *Yarrowia lipolytica*. J. Biotechnol. (submitted).

7) **Braga, A**.; Belo, I. (2014) Biocatalysis in micellar systems. *In* White Biotechnology for Sustainable Chemistry. Royal Society of Chemistry (accepted).

POSTER PRESENTATIONS

- **Braga, A**.; Gomes, N.; Belo, I. (2011) Lipase-mediated hydrolysis of castor oil on its biotransformation into γ-decalactone by *Yarrowia lipolytica*. 1st European Congress of Applied Biotechnology, September 25-29 Berlin, Germany.

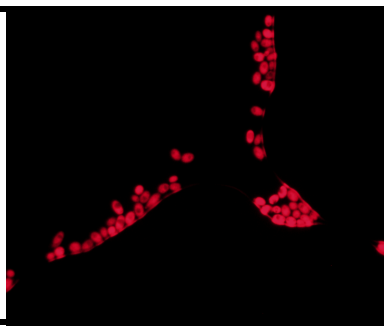
- **Braga, A**.; Gomes, N.; Belo, I. (2011) Strategies for increasing aroma production by *Yarrowia lipolytica*: hydrolysis of substrate and cell immobilization. Livro de Actas do Congresso MicroBiotec 11, 1-3 December, Braga, Portugal. p.109. ISBN:978-989.97478-1-4.

- **Braga, A**.; Belo, I. (2013) Increasing γ-decalactone production from castor oil by *Y. lipolytica* in a bioreactor by operational factors selection. 2nd European Congress Applied Biotechnology (ECAB). 21-25 April in The Hague, The Netherlands.

- **Braga, A**.; Belo, I. (2013) Enhancement of castor oil bioytransformation into aroma by *Yarrowia lipolytica* mutants. MicroBiotec' 13. 6 – 8 December, Aveiro, Portugal.

- **Braga, A**.; Crutz-Le Coq, A.M.; Dulermo, R.; Nicaud, J.M.; Belo, I. (2014) Comparison of γ-decalactone production from castor oil by *Y. lipolytica* mutants in batch and step-wise fed-batch cultures. 7th International Congress on Biocatalysis. August 31 – September 4. Hamburg, Germany.

LITERATURE REVIEW



γ-Decalactone is a peach-like aroma compound that is widely used in food and beverages, reason why the food industry is in an urge to find a cost-effective and nature-identical production for this compound. One of the best-known methods to produce γ-decalactone is from ricinoleic acid catalysed by *Y. lipolytica,* a GRAS (Generally Regarded As Safe) status yeast.

In this Chapter, the focus is the aromatic compound γ-decalactone and its production in by yeast *Y. lipolytica*. The metabolic pathway of lactone production and degradation are also addressed. Moreover, a brief overview about cell immobilization and biotransformation in biphasic systems is presented.

2.1 THE AROMATIC COMPOUND γ-DECALACTONE

The consumer demand for tasty foods has been growing, leading to an increasing need of aroma additions to replenish or add flavour to products. As a result, the production of these aromas, to be used by industrial companies such as food and beverages, soaps, cosmetics, chemical, pharmaceutical among others, has grown exponentially (Maracos and Schmidt-Dannert, 2003).

For a long time, the source of natural flavours were essential oils from higher plants, fruit juices, vegetables extracts and a very few selected products of animal origin (Krings and Berger, 1998). However, theses extractions have a low yield and its purification is often difficult making the overall process too expensive. Also, seasonal variations, political and socioeconomic factors lead to a constant shortfall of product supply of natural fragrances from natural sources (Longo and Sanromán, 2006). Chemical synthesis is an attempt to liberate the aroma industry from a dependence upon these factors, producing most of the today's existing flavours that are described as "nature-identical". Nevertheless, chemical synthesis generally requires numerous steps, often results in an environmentally unfriendly production process and in undesirable racemic mixture compounds (Gopinath et al., 2008; Vandamme and Soetaert, 2002).

The use of biocatalysts in the production of flavouring compounds similar with those present in natural sources is preferred, among others, due to regulatory reasons. In the U.S and according to European regulations (e.g. CFR 1990 and EEC 1334/2008), compounds isolated from natural resources or obtained in microbial or enzymatic processes involving precursors isolated from nature are classified as "natural". Consumer preferences reflecting the trends towards "health lifestyle" decide that the vast majority of flavour additives to be used in the food industry are compounds classified as "natural". Biotechnological production is an interesting approach for flavour production and has attracted a great deal of research interest (Longo and Sanromán, 2006).

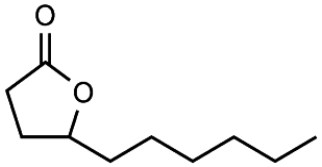
The aromatic compounds, especially lactones, are widely used in food industry. Lactones are molecules comprising a carbon cycle with one oxygen atom, resulting in a hydroxy acid cyclisation. These compounds are very attractive for the food industry since they have a very characteristic "fruity" aroma and is naturally found in a wide variety of foods (fruits, milk and dairy products, meats and some fermented foods) (Marasco and Schmidt-Dannert, 2003). For a long time they were obtained directly from fruits or by chemical synthesis, but over the past few years, the use of microorganisms

and enzymes for the production of natural flavour compounds has been extended (Endrizzi-Joran et al., 1993). The most important lactone for flavour application is γ-decalactone, with a mundial market volume of several hundred tons per year and it has an oily-peach aroma, an extraordinarily tenacious odour and a very powerful, creamy-fruity, peach-like taste in concentrations below 5 mg L^{-1}. The price of γ-decalactone was 20 thousand US$ kg^{-1} before the development of biotechnological methods of its synthesis which lead to a price reduction to 1.2 thousand US$ kg^{-1} (1986) that reached 500 US$ kg^{-1} in 1998 and 300 US$ kg^{-1} in 2004 with optimizations of the process (Schrader et al., 2004; Waché et al., 2003).

The interest of using yeast biotechnology for the production of lactones arose in the 60's, after results obtained by Okui et al. (1963) when studying the catabolism of hydroxylated fatty acids in several organisms. After that, numerous studies have been made on γ-decalactone production from yeast, often focused on the screening of yeast strains and medium optimization (Endrizzi-Joran et al. 1996). Most of the industrial processes use ricinoleic acid, the main fatty acid (about 90%) of castor oil, or esters thereof, for γ-decalactone biotechnological production. This aroma can be obtained from the biotransformation of ricinoleic acid, catalysed by enzymes present in microorganisms with GRAS status, conferring this way a natural label to the compound.

γ-Decalactone is a cyclic ester which results from the condensation of the alcohol group -OH and a carboxylic acid group -COOH of the same molecule. It is characterized by a closed ring consisting of four carbon atoms and a single endocyclic oxygen atom, coupled with an adjacent ketone; its molecular weight is 170 g mol^{-1} (Aguedo, 2002a). Some physical chemical properties of the γ-decalactone are presented in Table 2.1 (Souchon, 1994).

Table 2.1 – Physical chemical properties of the γ-decalactone (Souchon, 1994)

Physical chemical properties	Value	Structure
Volatility index	0.103	
Solubility in water	0.6 g L^{-1}	
Hydrophobicity constant at 25 °C	3.35	
Estimated saturation vapor pressure at 25 °C	0.754 Pa	
Boiling point	281 °C	
Diffusion coefficient:		
- in Water	6.6×10^{-10} m^2 S^{-1}	$(C_{10}H_{18}O_2)$
- in Methyl ricinoleate	0.11×10^{-10} m^2 S^{-1}	

There are several microorganisms selected for their potentialities to produce aroma, in which the most important are *Pseudomonas*, *Sporobolomyces*, *Pichia*, *Candida* and *Rhodotorula*, being *Y. lipolytica* species the one with a higher productivity. Table 2.2 presents a short summary of the microorganisms described in the literature, capable of producing γ-decalactone and respective aroma concentrations attained.

Table 2.2 - Microorganisms able to produce γ-decalactone

Microorganism		[γ-decalactone] (g L^{-1})	Authors
Bacteria	*Pseudomonas*, *Bacillus*, *Xanthomonas* or *Acetobacter*	c.a 6.6	Gocho et al. (1998)
Yeast	*Candida albicans, C. guilliermondii, C. krusei, C. parakrusei, C. pseudotropicalis, C, rugosa, C. stellatoidea, C. tropicalis*	0.2 to 0.86	Farbood and Willis (1983); Farbood et al. (1990)
	Hansenula saturnus	0.2 to 0.86	Farbood and Willis (1983); Meyer (1993)
	Pichia guiliermondi	0.1 to 0.5	Endrizzi-Joran et al. (1993); Iacazio et al. (2002)
	Sporidiobolus ruinenii, Sp. salmonicolor; Sp. pararoseus, Sp. johnsonii	0.012 to 5.5	Dufossé et al. (1998); Feron et al. (1996a)
	Sp. odorus	0.08 to 0.2	Cheetham et al. (1993); Lee and Chou (1994);
	Yarrowia lipolytica	0.2 to 12	Aguedo (2002a); Ambid et al. (1999); Farbood and Willis (1983; 1985); García et al. (2009); Gomes et al. (2007; 2010; 2012) Groguenin et al. (2004); Meyer (1993); Nicaud et al. (1996); Pagot et al. (1998); Rabenhorst and Gatfield (2000); Rabenhorst and Gatfield (2001); Reis et al. (2006); Waché et al. (1998; 2000a; 2000b); Wang et al. (1999)
	Rhodotorula aurantiaca	4.5 to 6.5	Alchihab et al. (2009); Alchihab et al. (2010)
Filamentous fungi	*Mucor sp.*	10.5	Kumin and Munch (1998)
	Trichoderma harzianum	0.26	Serrano-Carréon et al. (1997)

2.2 *YARROWIA LIPOLYTICA*

Yarrowia lipolytica is an aerobic, eukaryotic, non-pathogenic microorganism belonging to the Fungi kingdom (class Ascomycetes, subclass Hemiascomycetes). Initially, *Y. lipolytica* was classified as a species of the genus *Candida* once no sexual stage has been observed, more specifically as *Candida lipolytica* (Barth and Gaillardin 1997). The perfect form of *C. lipolytica* was identified at the end of 60 year (Wickerman et al., 1970) and *C. lipolytica* was renamed *Endomycopsis lipolytica* and *Saccharomycopsis lipolytica* (Yarrow, 1972) and finally *Yarrowia lipolytica* (van der Walt and von Arx, 1980). The generic name *Yarrowia* was proposed by van der Walt and von Arx (1980) in acknowledgement of a new genus identified by David Yarrow from the Delft Microbiology Laboratory (Yarrow, 1972). Deriving from the ability of this yeast to hydrolyse lipids, the specie was named "lipolytica".

This microorganism is one of the most extensively studied from the "non-conventional" yeast species (Barth and Gaillardin, 1997) and it is rather different from the well-studied yeasts models *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* regarding phylogenetic evolution, physiology, genetics and molecular biology (Wolf, 1996).

Yarrowia lipolytica is a dimorphic microorganism capable to grow in two distinct morphological forms, usually as single oval cells or as filamentous hyphae, being reversible between the two forms. It is believed that the dimorphism of this yeast, as well as other species, provides a mechanism for responding to adverse conditions (Kawasse et al., 2003). The morphology of the cells is strongly influenced by the growth conditions (aeration, carbon and nitrogen sources, pH, dissolved oxygen concentration in the medium, etc.) and by the genetic characteristics of the strain (Cruz et al., 2000). This yeast exhibits various colony shapes ranging from smooth and glistening to heavily convoluted and mate. Most strains are unable to grow above 32 °C (Holzschu et al., 1979) and its susceptibility to genetic manipulation and efficient transformation systems make this host an appropriate model for the study of dimorphism in yeasts (Szabo, 2001).

From mid-1960s ,*Y. lipolytica* has received a considerable attention because of its ability to use hydrophobic substrates such as *n*-alkanes, fatty acids, fats and oils. This attention leads to the development of molecular biology and genetic tools that improves the robustness of this organism and has guaranteed a spot as a profitable industrial organism. Nowadays, it is also used as a model organism for several studies of academic interest, like the degradation of *n*-alkanes (Fickers et al.,

2005), accumulation of lipids (Beopoulos et al., 2008), biosynthesis and degradation of peroxisomes (Gunkel et al., 1999), secretion of proteins and metabolites (Beckerich et al., 1998; Madzak et al., 2004; Nicaud et al., 2002), stress response (Kawasse et al., 2003; Biryukova et al., 2006), dimorphism (Chang et al., 2007; Titorenko et al., 1997, 2000), alternative intron splicing (Dujon et al., 2004), genome evolution (Gaillardin et al., 2012), and analysis of mitochondrial respiratory chain complex I (Ahlers et al., 2000; Angerer et al., 2012; Barth et al., 2003; Brandt et al., 2005; Kerscher et al., 2004). Properties like intracellular accumulation of oil, production of hydroxyl or dicarboxylic acids, as well as secretion of large amounts of organic acids focus the interest on this yeast as a potential producer of basic commodities, fine chemicals, or building blocks for chemical industry in the post-alkane area. Furthermore, the high capacity for proteases secretion and lipases favours this yeast as an enzyme producer as well as other heterologous proteins (Barth, 2013).

Carbon sources such as glucose, galactose and mannitol (Kreger van Rij, 1984), organic acids (lactate and citrate), amino acids (methionine), glycerol (Papanikolaou and Aggelis, 2003) and ethanol (Barth and Gaillardin, 1997) can be used by *Y. lipolytica*. Besides those, these species can utilise hydrophobic substrates such as alkanes (hexadecane, decane) and *n*-paraffins, lipids such as fatty acids (ricinoleic acid, palmitic acid, lauric acid) and triglycerides (trinoleine, tripalmitin) (Kreger van Rij, 1984) (Figure 2.1).

Yarrowia lipolytica can be found in several habitats such as soil and manure, but it can be also isolated from foods rich in fat and protein such as cheese, yogurt, meats and olive oil (Barth and Gaillardin, 1997). There are also *Y. lipolytica* strains isolated from polluted environments by petroleum or oils and agro-industrial wastewater (Mafakher et al., 2010).

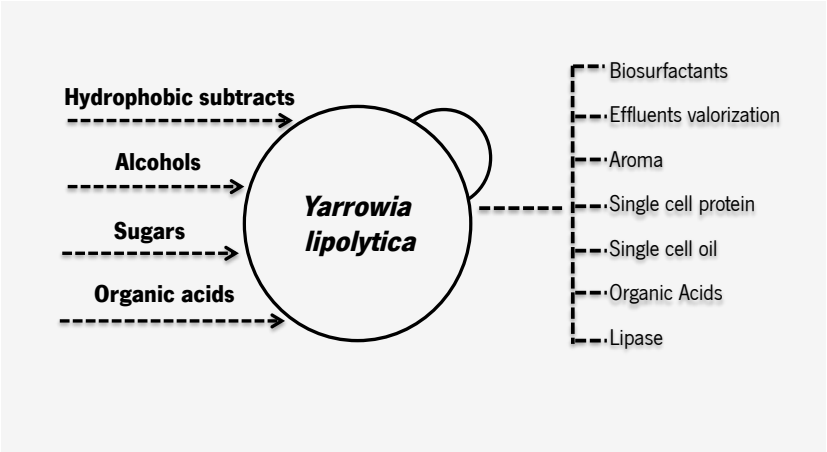


Figure 2.1 - Biotechnological applications of *Yarrowia lipolytica*.

2.3 γ-DECALACTONE PRODUCTION THROUGH PEROXISOMAL β-OXIDATION

The possibility of producing lactones using a biotechnological route was discovered in the 60's by Okui et al. (1963) during the studies of the hydroxyacids catabolism from several microorganisms. β-oxidation pathway is the classical biochemical route involved in fatty acids degradation. It acts on an acyl-CoA molecule and consists of four-step reaction sequence, yielding an acyl-CoA, which has two carbons less and an acetyl-CoA. This sequence is repeated several times until the complete breakdown of the compound (Fig. 2.2).

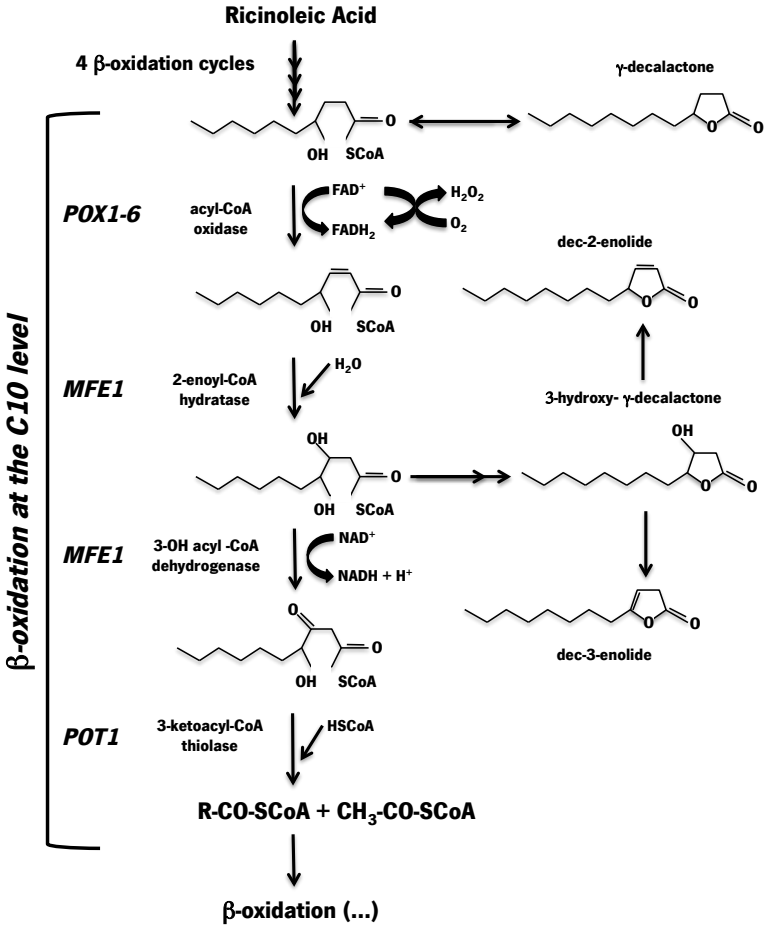


Figure 2.2 - The pathway from ricinoleic acid to γ-decalactone and enzymes involved in the yeast peroxisomal β-oxidation (Adapted from Waché et al. (1998) and Blin-Perrin et al. (2000)).

Lactonisation can occur at the whole C_{10} stage resulting in other decalactones of variable interest, dec-3-enolide, exhibiting very powerful fruity notes, and dec-2-enolide, characterized with mushroom notes (Gatfield et al. 1993). These lactones are probably related to a deficient 3-hydroxyacyl-CoA dehydrogenase activity. This later activity reduces NAD^+ to NADH which is regenerated through a shuttle mechanism (Hettema and Tabak, 2000), that probably depends on the mitochondrial respiration. The accumulation of these lactones is observed in anoxic environments provoked by high cell density. However, some fluctuations in the accumulated amounts are observed according to the chosen mutant (Waché et al., 2001).

The enzymes involved in this pathway usually work in several β-oxidation cycles and with different chain lengths metabolites. Depending on many factors, the breakdown can be stopped before the theoretical end, liberating medium- or short-chain volatile compounds. These metabolites can exit the pathway at each two β-oxidation cycles or inside the sequence, leading to a variety of volatile compounds. The commonly accepted pathway from ricinoleic acid to γ-decalactone is presented in Fig. 2.2: four β-oxidation cycles occur, yielding 4-hidroxy-decanoyl-CoA, which is then cyclised to γ-decalactone. The yeast *Y. lipolytica* possesses a family of six acyl-CoA oxidases (Aox1 to 6 encoded by *POX1* to *POX6*) (Fig. 2.3). The first enzyme of the pathway is generally considered as the limiting step in the catalysis (Fig. 2.3B) (Groguenin et al. 2004) and the role comprising the other acyl-CoA oxidase were enlightened with the mutations in the *POX* genes. The disruption of *POX1* resulted in an increased β-oxidation activity but a decrease on the production of γ-decalactone (Pagot et al. 1998). Two Aox exhibited a high activity and a chain-length specificity, one being long-chain-specific (Aox2) and the other short-chain-specific (Aox3). The role of the other Aox was less evident. Aox4, Aox5 and Aox6 exhibited a weak activity in the whole spectrum of straight-chain acyl-CoA (from C_4 to C_{18}) and Aox1 did not exhibit any detectable activity. The disruption of the genes corresponding to these three Aox resulted in a 2- to 5-fold increase in the global Aox activity, suggesting a role in the regulation of their activity. Also, in some *POX* mutants β-oxidation of C_{10} or smaller acyl-CoA is responsible for a decrease in the yield as a consequence of the γ-decalactone degradation (Waché et al., 2002; Groguenin et al., 2004).

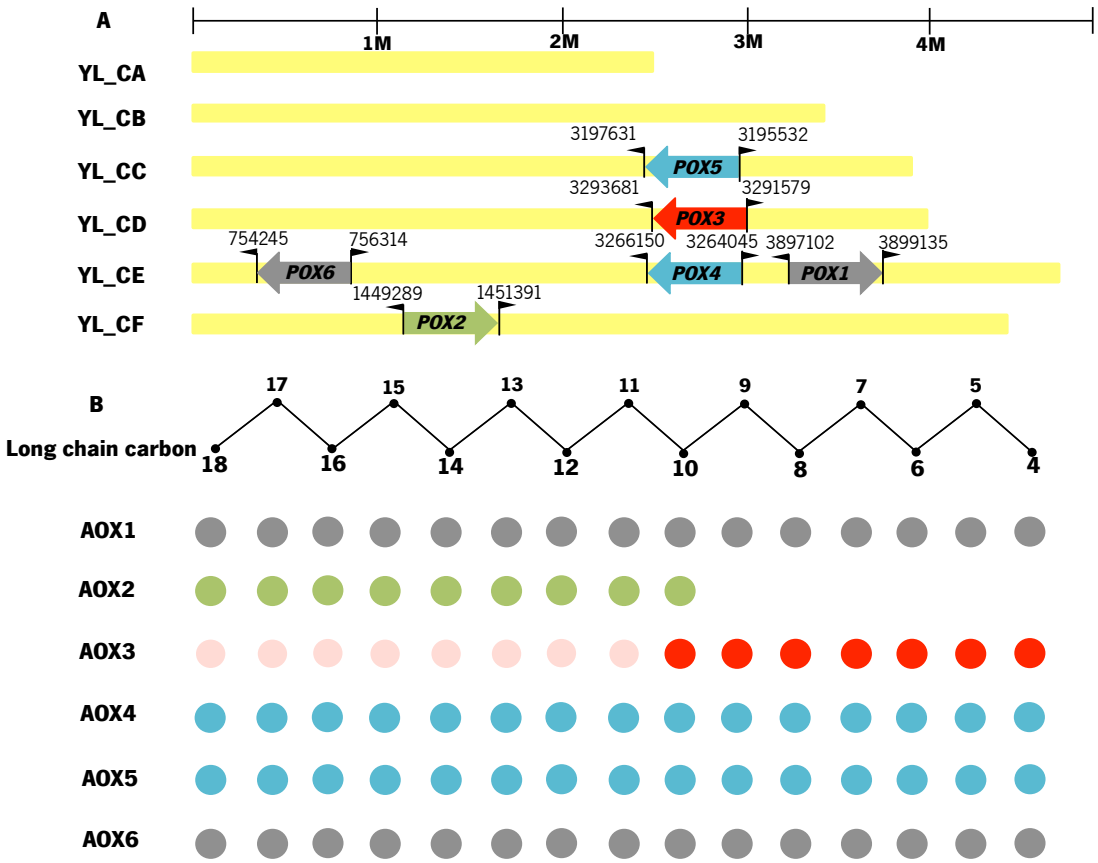


Figure 2.3 - Schematic representation of *POX* genes in *Y. lipolytica* genome and respective activity for the AOX enzymes. (A) Genes positioned in the different chromosomes (genes are not scaled). Color code is as in B. The 1M represents 1 million base pair. (B) Selective activity of AOX enzymes towards fatty acids of different chain length. The number of carbons of the substrate molecule is indicated in the top. The circles below indicate the qualitative activity for each AOX on the castor oil. Circles are filled with a colour code and indicate the activity level of the AOX towards the elongation of fatty acids: red - short chain fatty acids (the low intensity represent low activity for long chain fatty acids); green - long chain fatty acids; blue - whole spectrum of straight-chain; grey - not exhibit any detectable activity (Adapted from Groguenin et al. 2004, Waché et al. (2000b, 2001, 2002)).

Understanding the specific roles of each acyl-CoA oxidase has been the basis for the "construction" of strains growing at a good rate and producing γ-decalactone without degradation (Groguenin et al., 2004).

2.3.1 γ-DECALACTONE DEGRADATION PATHWAY

In the biotransformation of γ-decalactone from ricinoleic acid, a maximum concentration can be reached after which starts to gradually decrease probably due to degradation and/or re-consumption of the compound. Therefore, the γ-decalactone concentration in the medium results from the difference between what is produced and what is degraded (Endrizzi-Joran, 1994). This decrease in the aroma compound concentration may be extremely prejudicial to the productivity of the process and it has been observed for different yeast strains, such as: *Candida guilliermondii* (Okui et al., 1963), *Sporidiobolus salmonicolor* (Dufossé, 1993), *C. intermedia* (Endrizzi-Joran and Belin, 1995) and *Y. lipolytica* (Aguedo et al., 2005b) (Table 2.2).

Although the metabolic pathways of lactone degradation are not fully elucidated, there are some aspects related to this degradation that can be mentioned. First, the hydroxy acid form (unlactonised form) appears to be degraded faster than the lactone form (Endrizzi-Joran and Belin 1995), suggesting that the step of hydrolysis of the lactone exhibits a high control on the consumption. Then, the degradation pathway is very specific to the lactone (Fuganti et al. 1993; Latrasse et al. 1993; Fantin et al. 2001), since no degradation has been observed in the same conditions for lactones with similar structures, such as 3-hydroxy-γ-decalactone or decen-4-olides (Waché et al. 2001).

Several pathways of degradation are possible. The most probable one includes the opening of the cyclic form through a blood γ-lactonase activity (Fishbein and Bessman 1966), followed by the activation of the CoA esters and β-oxidation. When the hydroxy group is in the α-position, a α-decarboxylation is required prior to the β-oxidation (Voet and Voet 1990). Another possible pathway involves the ω-oxidation of the lactone to yield, after delactonisation, a ω-dicarboxylic acid. The production of such diacids by cells with the inability to perform β-oxidation reactions has already been described (Picataggio et al. 1992; Fabritius et al. 1998). Nevertheless, this behaviour is not restricted to cells lacking the enzymes to perform β–oxidation reactions, although in cells exhibiting an intact β–oxidation the substrate used are the dicarboxylic acids to envisage its degradation. The involvement of β-oxidation in the degradation is highly suggested by results obtained with acyl-CoA oxidase-modified mutants, in which the mutant lacking the enzyme of the β-oxidation pathway is the only one unable to degrade γ-decalactone (Waché et al. 2001).

2.4 NEW INSIGHTS INTO γ-DECALACTONE PRODUCTION

2.4.1 METABOLIC ENGINEERING OF β-OXIDATION PATHWAY

Developments for the lactone production processes have been made with the wild-type strain resulting in γ-decalactone concentrations of 12 g L^{-1} (Rabenhorst and Gatfield, 2000). Nevertheless, rapid lactone degradation is observed due to high activity level of the acyl-CoA oxidase in *Y. lipolytica*. Also, only a portion of the methyl ricinoleate is oxidized to the C_{10} level, and the C_{10} product serves as the precursor for other lactones (Farbood et al., 1989; Gatfield et al., 1993).

As an attempt to increase γ-decalactone concentration, serial knockouts for each acyl-CoA enzymes (Aox1 - 5) were made (Fig. 2.3) (Wang et al., 1999; Luo et al., 2000; Luo et al., 2002). Waché et al. (2000b, 2001, 2002) studied the involvement of these enzymes in the biotransformation of γ-decalactone by *Y. lipolytica* and built strains that were disrupted in one or several acyl-CoA genes. They observed that the strain disrupted for *POX2*, *POX3* and *POX5* (which still possess *POX4,* encoding a weakly active Aox) and with *POX2* gene reincorporated in a plasmid, produced more lactone which is not consumed. Also, Groguenin et al. (2004) constructed a mutant strain (Δ*pox2-pox5,* pPOX2-*POX2*) that produced about 10 times more γ-decalactone than the WT (400 mg L^{-1} *vs* 100 mg L^{-1}) and was unable to degrade this aroma. Guo et al. (2011) reconstructed a mutant strain with *POX2* gene overexpressed and a knockout in the *POX3* gene*,* and observed that γ-decalactone production increased as a result of these two alterations and no aroma reconsumption was observed.

Another problem regarding γ-decalactone production is the switch between production of γ-decalactone and production of hydroxy-related lactones, which decreases the γ-decalactone productivity. Waché et al. (2001) observed that the accumulation of 3-hydroxy-γ-decalactone in the wild type was related with the high Aox efficiency which possesses six isoforms. The combined disruption of the Aox-encoding genes (like Δ*pox2pox3*) allows the reflux towards the production of γ–decalactone and the accumulation of hydroxy-lactone is no longer observed.

Nevertheless, the lactone concentration in the medium may also be a limiting factor for higher productivities, since these metabolites are known to be toxic to the producing yeasts (Feron et al., 1997). The decline in cell viability has been clearly associated with the increase in lactone

concentration within the culture media during biotransformation (Feron et al., 1997; Dufossé et al., 1999).

In the future, *in silico* genome-scale analysis will be an important tool to understand γ-decalactone production. The construction of a metabolic model of *Y. lipolytica* based on genome annotation data and biochemical parameters will be very important to find a non-straightforward solution. Indeed, using this kind of models it will be possible to predict which gene knock-out(s) or overexpression(s) will re-direct the flux towards the production of a desired compound. Overall, the model will be a valuable tool and a platform for the system biology analysis of *Y. lipolytica*, therefore actively contributing in the important metabolic basis and feasible ways to engineer the improvement of γ-decalactone synthesis. Besides taxonomic and evolutionary studies the increasing knowledge of *Y. lipolytica* metabolic pathways along with the existence of sophisticated genetic tools will offer new perspectives towards its biotechnological applications. However, very few studies concerning this topic were been performed. Loira et al. (2012) combined *in silico* tools with manual curation to produce the first genome-scale metabolic model for *Y. lipolytica* (*Y. lipolytica* iNL895). After that, Pan and Hua (2012) have reconstructed this model combining genome annotation and a more detailed biochemical knowledge, *i*YL619_PCP.

2.4.2 BIOTECHNOLOGICAL DEVELOPMENTS

2.4.2.1 BIOTRANSFORMATION IN BIPHASIC SYSTEMS

Besides the advances in the construction of modified strains which allowed the production of higher γ-decalactone concentrations in the culture medium, the biotransformation of ricinoleic acid with *Y. lipolytica* has gained special attention from researchers in many different aspects.

Ricinoleic acid is the precursor used in γ-decalactone production. In some cases hydrophobic substrates like castor oil, fatty acids or esters of these compounds (Page and Eilerman, 1996), as methyl ricinoleate, are used as substrates.

Several studies were published regarding the toxic effect of the substrates and the lactone on the producing organism, improvement in the process productivity, and in the production of aroma at a large-scale level. In these studies, castor oil was normally used as substrate due to its large availability compared with other sources (e.g. methyl ricinoleate) and for its lower price. Castor oil is obtained by

extraction from the seed of a plant called *Ricinus communis* that belongs to the family *Euphorbiacae* (Kirk, 1979). This pale yellow colored oil is cheap and eco-friendly. It is nonvolatile and nondrying in nature and has a characteristic odor. From the triglycerides present in castor oil, glycerol is esterified at a level of approximately 90 % by ricinoleic acid, a unique hydroxylated, monounsaturated 18-carbon fatty acid (Naughton, 1979). The other most relevant fatty acids in castor oil are linoleic acid (4 %), oleic acid (2 %), stearic acid (1 %), palmitic acid (1 %), dihydroxystearic acid (0.7 %), linoleic acid (0.3 %) and eicosanoic acid (0.3 %). This substrate has the peculiarity of being practically insoluble in water and therefore forms a second phase. To facilitate contact between the yeast presented in the aqueous phase and the substrate, an emulsion stabilized by a surfactant can be formed.

The emulsifying agents or surfactants constitute an important class of chemical compounds used in various industrial sectors (Nitschke and Pastore, 2002) and can be synthetic or biologically produced (Amaral et al., 2006a). Surfactants are amphipathic molecules consisting of a non-polar hydrophobic group and a polar hydrophilic group. The non-polar group is often a hydrocarbon chain, while the polar group can be ionic (anionic or cationic), non-ionic or amphoteric (Nitschke and Pastore, 2002). They tend to be distributed at the interface between liquid phases with different degrees of polarity (oil-in-water and water-in-oil). The formation of a molecular film, placed at the interface, reduces the interfacial and surface tensions, being responsible for the unique properties of surfactants (Nitschke and Pastore, 2002). Aguedo (2002a) tested several surfactants (Tween 80, Triton X-100 and Saponin with neutral character; SDS, with anionic character; and CTAB, with cationic character) to determine their effects on: 1) viability of the yeasts, 2) membrane interaction, 3) emulsion, 4) hydrophobicity of yeasts surface and 5) biotransformation. The author concluded that Tween 80, a non-ionic surfactant derived from olyethoxylated sorbitan and oleic acid was the ideal surfactant, since it was the compound that allowed achieving higher γ-decalactone concentrations. Moreover, it had no effect on cell viability and no interaction with their membranes, providing a greater interfacial surface to the medium and the largest relative surface hydrophobicity to the cells. So, the interfacial area between the organic and aqueous phases becomes important in favouring the access of cells to the substrate. The lipid phase is dispersed as droplets in the aqueous phase (oil-in-water emulsion), presenting a large interface between the two phases. This involves another phenomenon which is the adhesion between lipids and microbial cells.

Yarrowia lipolytica cells are hydrophilic with a good attraction to hydrophobic surfaces or molecules when previously immersed in water. Bakhuis and Bos (1969) observed that the transfer between the lipid phase and microorganisms does not depend on the size of the fat globules but on the

size of cells. The growth rate is minimal when the two are about the same size and maximal when the droplets are smaller or larger than the cells. During the biotransformation of castor oil by *Y. lipolytica* direct contact occurs between the surface of the cells and the small substrate droplets and it is possible to increase the contact by choosing a surfactant having an affinity for the yeast (cationic surfactant) (Aguedo et al., 2004). The interaction between the cells and the hydrophobic surfaces or molecules is mediated by proteins or glycoproteins of the cell wall (Amaral et al 2006a). However, the cell surface properties may be intentionally modified in order to improve the cell-substrate adhesion. The surface charges may be altered, for example by modifying the medium pH or ionic force. Pre-culturing *Y. lipolytica* on a hydrophobic carbon source rather than on the classical glucose medium increases the subsequent adsorption of droplets on the cell surface (Aguedo et al., 2003). The addition of a chosen charged surfactant to the medium enables to obtain lipid droplets with a controlled-charge and a consequent optimised adhesion (Ravaine et al., 2002).

The hydrophobicity of the cell surface is considered one of the most important factors in the assimilation of hydrophobic compounds (Kim et al., 2000). Aguedo et al. (2005b) studied the effects of the cells surface charges on the biotransformation of methyl ricinoleate into γ-decalactone. The ζ values of the substrate droplets were modified by adding a cationic surfactant into the medium at concentrations that did not diminish cell viability, observing that the adhesion of the lipid substrate to the cells was increased but the overall performance of the process maintained, concluding that the adhesion is not rate limiting here. Observations of *Y. lipolytica* during the biotransformation of methyl ricinoleate showed that the contact occurs mainly through the adhesion of small-sized droplets (with diameters less than 2.5 μm) on the surface of the yeast. Further investigations showed that these adsorptions are mediated in part by Lewis acid-base interactions rather than by hydrophobic interactions (Aguedo et al., 2003). Electrostatic forces, and thus the surface charges of the cells and of the droplets, are also implicated in the adhesion between both entities. This fact was also observed by Waché et al. (2000a) during *Y. lipolytica* growth in methyl ricinoleate. Oil droplets had a much smaller size than yeast cells and the substrate transfer to cells was facilitated in this conditions.

In γ-decalactone production, high concentrations of aroma compounds reached during the biotransformation may lead to toxic effects towards the producing microorganism (Dufossé et al., 1999). Yeast cells are able to degrade γ-decalactone, however, the compound becomes toxic at a certain concentration threshold. Studies concerning the mechanisms leading to lactone toxicity showed that the hydrophobic lactone could take part in the cell membranes and increase membrane fluidity. γ-Decalactone concentrations higher than 150 mg L^{-1} lead to a dissipation of cell membrane potential

(Aguedo et al., 2003; Aguedo et al., 2002a). Retention of aroma compounds within cell membranes and a consequent toxic effect, thus needs to be considered for those compounds with hydrophobic properties. In order to reach high concentrations of the metabolites in the medium, different strategies need to be used taking into consideration the "cell-membrane-fluidising" action of the compounds (Aguedo et al. 2002b).

2.4.2.2 OPERATION IN BIOREACTOR

- Bioreactors

Cell viability, activity and growth are important parameters to take into consideration when choosing a method for the cultivation of microorganisms. During the biotransformation of castor oil in γ-decalactone by *Y. lipolytica,* an emulsion is formed where the fat globules and the cells are suspended. It will, therefore, be necessary to disperse the cells evenly in the medium, with air and agitation. Some bioreactors have been used for lactone production as an attempt to prevent the problems with medium emulsification.

Most of the studies describe aroma production in agitated Erlenmeyer flasks and depending on the agitation, the culture medium may be more or less agitated and aerated. In order to improve the process performance, baffled Erlenmeyer flasks are used since its design increases the turbulence of the culture medium and improves the efficiency of oxygen transfer. However, these systems do not allow to control of the aeration or agitation separately (Gatfield, 1988; Okui et al., 1963; Serrano-Carreon et al., 1997).

The stirred tank bioreactor is the most common reactor used in fermentations and cell cultivations. Usually, in these bioreactors, air is injected at the bottom of the tank and a Rushton turbine is normally located immediately above the air injector in order to reduce the bubbles size and increase the oxygen transfer rate to liquid phase. Also, it is possible to control agitation and aeration rates to maintain a constant dissolved oxygen concentration in the medium. Nowadays, most of papers describing γ-decalactone production in large-scale use stirred tank bioreactors. Feron et al. (1996b) compared the γ-decalactone production by *S. salmonicolor* and *S. ruinenii* in a 7 L stirred bioreactor using 41 g L^{-1} of methyl ricinoleate. The cultures were stirred (250 rpm) and aerated (1 vvm) with a volumetric oxygen transfer coefficient of 90 h^{-1} leading to aroma production of 1.6 g L^{-1} after 6 days of culture.

After studies of Belo et al. (1996) the use of pressure bioreactors were also presented as a good alternative for oxygen mass transfer improvement by raising air pressure in the reactor that increases O_2 solubility (Yang and Wang 1992, Belo et al. 2003). Aguedo et al. (2005a) performed some studies applying this bioreactor for γ–decalactone production and observed that although cells grew normally under high-pressure, i.e., under increased O_2 solubility, γ-decalactone production decreased in these conditions.

Although stirred tanks are the most common industrial bioreactors used for aerobic fermentations, they are not the best design for microbial cultures, for several reasons: the degree of agitation required to achieve sufficient oxygen mass transfer sometimes causes damage to the microorganisms; the mechanical energy input is high (meaning high costs) resulting in overheating that has to be controlled. Furthermore, due to their complexity, the stirred reactors are more expensive and less robust than other types of bioreactors.

Based on these disadvantages other designs were investigated, among which airlift reactors (Chisti, 1989). Airlift bioreactor are pneumatically agitated and often employed in bioprocesses where gas-liquid transfer is important. Gas provide the contact with the liquid for mass transfer process such as absorption or desorption and to provide energy through gas expansion or bubble resistance for liquid mixing. In this bioreactor, gas is sparger usually through the bottom and the ascending of gas bubbles causes mixing. The liquid recirculation occurs due to the four distinct sections, the riser, downcomer, gas separator and bottom. Some attractive features of the airlift are the low power consumption, the simplicity of its construction with no moving parts, high mass and heat transfer rates and uniform distribution of shear (Chisti and Moo-Young, 1989; Merchuck et al., 1994). However, few studies concerning lactones production with this type of bioreactor were described in the literature (Gomes, 2011a; García, 2008).

- OPERATION MODE

In microbial processes three different modes of operation have been extensively applied which are the batch, fed-batch and continuous mode. In a batch process, all nutrients required during one run of cultivation are added to the medium before cultivation is started with exception for molecular oxygen in an aerobic process and ammonia or other chemicals for pH adjustment, and the final products are removed at the end of each batch run. In a continuous process, all nutrients are continuously added to the bioreactor and fractions of the medium are removed at the same flow rate

as the supplied nutrients are added, so the volume of the culture is kept constant. In addition to these "ideal" conditions many modifications can be introduced, concerning the mode of addition of raw material(s) and withdrawal of products, like fed-batch. This is defined as a technique where one or more nutrients are supplied to the bioreactor during cultivation and in which the products remain in the bioreactor until the end of the run (Kim et al., 1999). In some cases, all nutrients are gradually fed into the bioreactor. The process starts as batch but when the initial substrate is consumed, fresh medium starts to be fed to the bioreactor. The inlet substrate feed should be as concentrated as possible to minimize dilution (Saarela et al., 2003). The main advantages of the fed-batch operation over the batch process are the possibilities to control both reaction rate and metabolic reactions by substrate feeding rate, allowing achieving high cell density, which is often necessary for high yield and productivity of the desired product (Yamane and Shimizu, 1984). The fed-batch processes are also employed to prevent the inhibition of cells by substrate. Fed-batch cultures have been widely employed for the production of various bioproducts including primary and secondary metabolites, proteins and other biopolymers.

This feature is especially interesting for the process of aroma production, considering the toxicity of ricinoleic acid to the cells (Feron et al., 1996b; Lee et al., 1995; Lin et al., 1996; Lee et al., 1998; Lee et al., 1999a). With this approach, it is possible to supply more substrate to the cells, preventing the toxic effects of ricinoleic acid. The fed-batch operation mode is successfully applied in several bioprocesses involving *Y. lipolytica* (Fickers et al., 2009; Kim et al., 1999; Kyong and Shin, 2000; Nicaud et al., 2002; Rymowicz et al., 2009; Turki et al., 2010). However, there are a few studies using this culture strategy for γ-decalactone production (Kapfer et al., 1989; Kumin and Munch, 1998; Lee et al., 1995; Gomes et al., 2012; Moradi et al., 2013). Thus, this operation strategy can be a great alternative to increase γ-decalactone production. Nevertheless, in this case, the maintenance of an emulsion causes numerous constraints to ensure that the supply of fresh medium and withdraw concerns an emulsion with the same characteristics. Thus, the substrate addition by pulses (step-wise feed-batch) is a way of circunventing this problem (Lee et al., 1995; Kim and Hou, 2006).

- Oxygen mass transfer in biphasic systems

For bioprocesses in which oxygen is consumed, that is the case of γ-decalactone production by *Y. lipolytica*, the mass transfer within the system is of crucial importance, especially if O_2 transfer can become a limiting step for the overall productivity. In biotransformation of castor oil into γ-decalactone by this yeast this is very important, firstly because this yeast is an obligate aerobe, and secondly

because not only the oxygen concentration but also the global redox environment (Feron et al., 2002) may influence the crucial step leading to the formation of other C_{10} lactones from 4-hydroxydecanoic acid, the direct precursor of γ-decalactone (Waché et al., 2001). Since oxygen is more soluble in the organic phase (castor oil) than in the aqueous phase, increasing concentration of the oil leads to an improvement of the oxygen mass transfer, important parameter for the process efficiency. Oxygen transfer rate from the gas to the liquid medium can also be improved by increasing the aeration and stirring rates (Chistia and Jauregui-Haza, 2002).

Nevertheless, the addition of oil to an aqueous medium modifies its properties: the density, the viscosity and the solubility and diffusivity of O_2 are changed; consequently the access of the cells to O_2 may be altered. The presence of a dispersed oil phase in a continuous aqueous phase generally increases the oxygen mass transfer rates within the medium (Nielsen et al., 2003). However, for low concentrations of insoluble substrate, the presence of the organic phase can retard the gas to water mass transfer.

In a two-phase system bioreactor, mass transfer occurs in a series of steps, from gas to water and from water to oil. The presence of a surfactant in the medium could complicate the transfer, but this point has still to be evaluated. Moreover, there is a small amount of data regarding the subject of mass transfer from the gas phase to oil-in-water dispersions in biological systems during the growth of microbial cells, which can be considered as an additional solid phase.

The effect of the hydrophobic phase on k_La may vary according to the range of concentrations (Gomes et al. 2007). Studies conducted in pneumatic agitated bioreactors, like bubble columns and air-lift bioreactors, have shown that the increase of the hydrophobic fraction in an oil-in-water emulsion can decrease k_La, since it causes a decrease in the interfacial area (a) for mass transfer. This phenomena occurs as a consequence of the surfactant molecules partition that arises between water and oil phases, leading to the increase of air bubbles size and coalescence, thus decreasing the bubble dispersion in the liquid medium (Gómez-Díaz et al., 2009). The presence of hydrophobic compounds, like the substrates for γ-decalactone production (e.g. castor oil and methyl ricinoleate), in culture medium generally leads to a global enhancement of oxygen transfer rate. This improvement is mostly attributed to the high affinity of oxygen to these compounds, thus increasing the driving force for oxygen mass transfer from gas to the bulk liquid phases. Even if some other means are available to increase the transfer of oxygen within the bioreactor, such as the use of oxygen carriers like perfluorocarbons (Lowe, 2002) and silicone oils (Leung et al., 1997) or the use of hyperbaric air (Belo

et al., 2003) or pure oxygen as sparging gas, it seems important to understand better the mass transfer in multiphase systems in order to predict and optimize the oxygen availability for the aerobic cells.

2.4.3 CURRENT DEVELOPMENTS

The main topic in the strategies to improve the γ-decalactone productivity, as previously discussed, concerns the β-oxidation pathway (Pagot et al. 1998; Waché et al. 1998, 2000b, 2001; Blin-Perrin et al. 2000). Beside the genetic engineering approaches, it is also possible to modify the environmental conditions to change β-oxidation fluxes. To achieve this goal, a recent study utilised the reducing agent dithiothreitol in a *Sporidiobolus* strain to increase the γ-decalactone production (Wang et al. 2000). Although, the effect of this compound on the cell was not investigated, the amount of the lactone increased. Additionally, these authors have found out that by adding a phosphate buffer at pH 7, the concentration of the γ-decalactone in the medium increased 3-fold, in comparison with a non-buffered medium. Also, Gatfield et al. (1993) noted a strong impact of the agitation parameter on the production of the lactones.

Nevertheless, the highest drawback may arise from yeast sensibility and toxicity towards elevated concentrations of lactone which can be a limiting factor in the industrial implementation of this production (Feron et al. 1997). Some strategies have been developed to reduce lactone toxicity released in the fermentation medium, as for example the addition into the culture media of inert oils (hydrogenated coconut oil or a mixture of tripalmitine, tris- tearine and triolein) or hydrophobic porous sorbents (Dufossé et al. 1997, 1999; Souchon et al. 1998). The most common methods used to remove organic compounds from the fermentation medium involve solvent extraction, trapping in vegetable oils, adsorption on activated carbon or on porous hydrophobic polymers. β-Cyclodextrins were also used to extract numerous compounds by inclusion (Bar, 1989). Three techniques (*in situ* trapping in oily phases, inclusion in β-cyclodextrins, adsorption on porous hydrophobic sorbents) were studied to overcome the toxicity of γ-decalactone produced by *S. salmonicolor* using ricinoleic acid as precursor (Dufossé et al. 1999). Oily phases added to the media (olive oil, paraffin) have a protective effect on *S. salmonicolor*. β-Cyclodextrins were added in the culture medium (5 g L^{-1}) containing toxic concentrations of γ-decalactone (400 and 600 mg L^{-1}). The lactone trapping in cyclodextrins were shown insufficient for the preservation of cell viability. On the other hand, the adsorption of γ-

decalactone on activated carbon and porous hydrophobic polymers (Porapak Q, Chromosorb 105 and SM4) is a suitable method to extract this molecule from aqueous medium (Dufossé et al. 1999; Souchon et al. 1998). Dufossé et al. (1999) and Souchon et al. (1998) reported that the presence of adsorbents in the biotransformation medium allows maintaining concentration of lactone very low levels in the medium and so reduces the toxicity against the yeast.

Another important feature is the possibility to decrease the fluidising action of γ-decalactone on the cells by lowering the temperature of the cultivation. Within this subject, Alchihab et al. (2009) used a psychrophilic strain of the yeast *Rhodotorula aurantiaca* that produces high amounts of 4-hydroxydecanoic acid and γ-decalactone from castor oil (4.2 and 2.2 g L^{-1}, respectively) at 12 °C (Alchihab et al. 2010). Moreover, the use of different natural gums (gum arabic, gum xanthan and gum tragacanth) on the growth and production of γ-decalactone by *R. aurantiaca* were studied to reduce its toxicity towards the cells. Positive results were found for the gum tragacanth that was able to enhance growth of the yeast during the production of the compound (Alchihab et al. 2010).

A recent development in this area is the use of permeabilized cells, since the microbial production of lactone has a critical problem which is a low yield that results from the barrier effect of the cell wall or membrane (Kondo et al., 2000). Cell permeabilization improves the transfer of the reaction substrate and product across the cell membrane and thus increases the production of metabolites (Matsumoto et al., 2001; Chow and Palecek, 2004; Lee et al., 2004). An et al. (2013) increased γ-dodecalactone production by effectively transferring the substrate and product into cells, by permeabilization of *Waltomyces lipofer*. A new biotransformation process for the production of the natural flavour lactone was developed using permeabilized cells. γ-Dodecalactone production by the new process using permeabilized *W. lipofer* cells was significantly higher than using non-permeabilized cells and these cells displayed the highest concentration and productivity observed in the microbial production of the flavour lactone.

2.5 WHOLE - CELL IMMOBILIZATION

On traditional fermentations, the use of free cells suspended in the medium influences the process yield, namely the low cell density, nutritional limitations, potential toxicity of reactants and products and difficulties in the recovery of final products (Léonard et al., 2011). In the last decades,

the immobilization of cells has been addressed and arising as an alternative to the conventional methods of cultivation.

Immobilization of cells is the attachment of cells or their inclusion in a distinct solid phase that allows exchange of substrates, products, inhibitors, but at the same time separates the catalytic cell biomass from the bulk phase containing substrates and products. This strategy keeps high cell densities, protects cells against stress environmental conditions, prevents cells washout, improves reaction yields and rates, increases bioreactor volumetric productivity, increases the reusability of the biocatalyst at batch and continuous process and eliminates the time-consuming and expensive steps involved in isolation and purification of intracellular enzymes (Léonard et al., 2011; Park and Chang, 2000; Trelles et al., 2010). However, when a particular immobilization method is selected, it is crucial to take into consideration the immobilization costs, mass transport limitations, applicability of final product, ensure about cell activity and viability, among others (Zacheus et al., 2000).

2.5.1 IMMOBILIZATION METHODS

The selection of an immobilization technique is based on process specifications for the catalyst, including cost of immobilization procedure, toxicity of immobilization reagents, and the desired final properties of the immobilized cells (Malcata and Hill, 1991).

Adsorption, covalent bonding, crosslinking, entrapment, and encapsulation are some of the most described methods for immobilization (Kinoshita *et al.*, 1957).

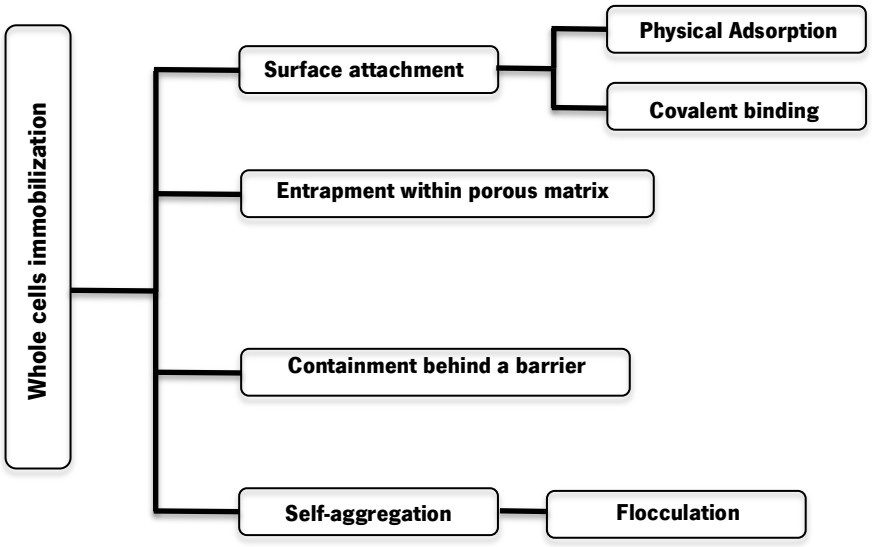


Figure 2.4 – Cells immobilization methods (Adapted from Batzias and Siontorou (2012) and Pilkington et al. (1998)).

At physical adsorption, interaction between microbial cells and the solid carriers occurs by electrostatic, ionic and hydrophobic interactions (Oliveira, 1997). Once the cells are not confined in the solid-supportive phase, the detachment of cells and its relocation is then possible. The attachment to a surface can be done by natural adsorption, electrostatic forces or covalent binding, using different cross-linking agents to achieve the cell immobilization. Van der Waals forces, electrostatic interactions and covalent binding, have an important part in the adsorption process (Margaritis and Kilonzo, 2005). Physical adsorption is used to immobilize microorganisms that are prone to adhere and multiply under some surfaces. The first method used for whole cells immobilization was reported by Hattori and Furusaka (1960) by binding of *Escherichia coli* cells onto an ion exchange resin. Beyond this carrier, a wide range of them have been explored and used, including microcarriers that are small-diameter beads from 100 – 200 μm that are manufacture using different synthetic polymers (polystyrene, polyamide and polyacrylamide), inorganic carriers (clay materials, silica, activated charcoal and metal oxides), biopolymers (cellulose and derivates, dextran, starch, collagen and gelatin), ionic exchangers (amberlite, Dowex DEAE-Sephadex and DEAE-cellulose), glass, wood, glass ceramic, plastic materials and ceramics (Batzias and Siontorou, 2012; Ramakrishna and Prakasham, 1999; Villeneuve et al., 2000). The surface of the immobilization support is important in the adsorption process of cells as rough surfaces allows the cell retention into the support cavities (Bránýik et al., 2004; Genisheva et al., 2011). This immobilization technique is often used for its easiness and spontaneity. However, there is no barrier between the liquid and the immobilized cells which facilitates the cell detachment from the support. Normally, the equilibrium between free and immobilized cells is established at some point of the cell growth. The detachment of cells depends on the age of the cell, cellular wall composition, pH and ionic composition of the medium. However, the desorption is compensated with the growth of new cells on the support (Strehaiano et al., 2006). The natural adsorption technique is advantageous over other types of immobilization as the oxygen transfer is good and no scale-up drawback exists (Ory et al., 2004).

The entrapment within porous matrix is the second most important technique of immobilization (Verbelen et al., 2006) and can be performed by two approaches: 1) cells are introduced in a porous material and, after growing, their mobility is restricted by the presence of other cells and by the matrix; 2) a solid matrix is synthesized *in situ* around the cells. The cells are incorporated in the matrix of a more or less rigid polymer, such as polyacrylamide, proteins (gelatine, collagens) and polysaccharides (cellulose, alginate, agar, carrageenan). This technique can be expensive and time consuming

(Verbelen et al., 2006), with serious drawbacks such as diffusion limitations of nutrients, metabolites and oxygen, as well as instability of the gel beads and detachment of cells (Kourkoutas et al., 2004). Calcium alginate gel is the most commonly used material for cell entrapment in the food industry (Strehaiano et al., 2006).

Containment behind a barrier can be achieved by two main methods: 1) entrapment of the cells in microcapsules; 2) by the use of microporous membrane filters (hollow fibre) or by cell immobilization onto an interaction surface of two immiscible liquids (Kourkoutas et al., 2004; Verbelen et al., 2006). The entrapment of cells in microcapsule or encapsulations, consists firstly in entrapping the cells in a spherical gel and posterior coating with a polymer such as polyethyleneimine. Then, the gel is dissolved but the cells are left in suspension, contained behind the polymer barrier. The microporous membranes filters are normally made of polymers, e.g., polyvinylchloride or polypropylene (Margaritis and Kilonzo, 2005). The containment of the cells behind a barrier allows very high cell concentrations. This method of immobilization is normally used when a cell free product is needed. The main disadvantages are related to mass transfer limitations and the possibility of membrane fouling caused by the cell growth (Gryta, 2002).

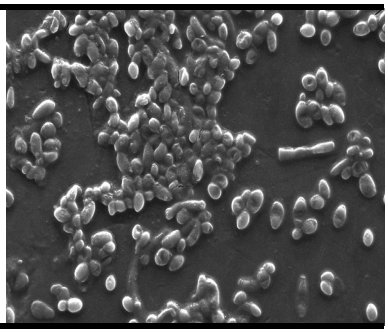
The flocculation is the formation of cellular aggregates in suspension, coupled with a rapid sedimentation in a natural manner or in the presence of flocculating agents. Thus, flocculation can be considered as one of the most promising techniques of restraint to be used on a large scale because of the increased potential of forming cellular aggregates in fixed bed reactors and fluidized or tanks with agitation (Freeman and Lilly, 1998). The flocculation method is normally used in the beverage industry, since the flocculation of *Saccharomyces cerevisiae* cells positively affects the fermentation time, facilitates the removal of cells and contributes to increase the beer quality (Hsu *et al*., 2001).

The use of immobilized cells in a matrix to produce aroma has theoretical problems of mass transfer (substrate, oxygen metabolites) still, this strategy can protect cells and in some cases increase the γ-decalactone production. Immobilization of cells by inclusion is the most common method being calcium alginate the most commonly used polymer due to its non-toxicity towards *Y. lipolytica* cells. Full description different carriers applied for *Y. lipolytica* immobilization and respective bioprocess used are compiled in Table 2.3.

Table 2.3 - Immobilization methods and carriers applied in *Y. lipolytica*

Strain	Bioprocess	Immobilization Method/ Carrier	Reference
Y. lipolytica	Citric acid biosynthesis using glucose as substrate	Alginate K-Carrageenan Polyurethane gel Nylon web Polyurethane foam	Kautola et al., 1991
Y. lipolytica EH 59	Citric acid biosynthesis using glucose and fructose as substrate	Microencapsulation in polyelectrolyte complex	Mansfeld et al., 1995
Y. lipolytica 180	Removal of oil films on surface waters	Oil-absorvent polyurethane	Oh et al., 2000
Y. lipolytica NCIM 3589	Crude oil degradation	Agar-Alginate composite beads	Zinjarde and Pant, 2000
Y. lipolytica W29	Oil waste water degradation	Calcium alginate	Wu et al., 2009

GENERAL METHODOLOGY



The general methods, operation conditions and equipment used in this work are presented in this chapter.

3.1 MICROORGANISMS, MEDIA AND CULTURE CONDITIONS

3.1.1 MICROORGANISMS

The yeast species used in the present work was *Yarrowia lipolytica*. In Table 3.1 the different strains studied are listed.

Table 3.1 - *Yarrowia lipolytica* strains used in this work

Strain	Genotype	Reference
W29 (ATCC 20460)	Wild type (WT)	- - -
MTLY40-2P	*Δpox2 Δpox3 Δpox4 Δpox5* + pPOX2*- *POX2*	Groguenin et al. (2004)
JMY3010	W29 derivative containing an additional copy of *LIP2,* pTEF-*LIP2*	Kindly supplied by Prof.° Nicaud, MICALIS (INRA-AgroParisTech)

* The pPOX2 promoter contained a deletion that results in a decrease catabolic repression (Nicaud et al., 2002)

3.1.2 YEAST PRESERVATION

Yarrowia lipolytica strains were stored at – 80 °C in cryogenic tubes (Microbank, Pro-Lab Diagnostics, Canada). After thawing, each strain was cultured for 48 h on YPDA medium (30 g L^{-1} agar, 20 g L^{-1} glucose, 20 g L^{-1} peptone, 10 g L^{-1} yeast extract) at 27 °C. The colonies were stored at 4 °C, to be used posteriorly to inoculate the culture media.

3.1.3 CELL GROWTH MEDIUM

Unless otherwise stated, the cell colonies previously prepared were used to inoculate (cell density of 0.5 g L^{-1}) a 500 mL baffled Erlenmeyer flask containing 200 mL of YPD medium (glucose 20 g L^{-1}, peptone 20 g L^{-1} and yeast extract 10 g L^{-1}). The medium was sterilized in an autoclave, at 121°C during 20 minutes.

Flasks were incubated at 140 rpm and 27 °C for 21 h until the total glucose consumption, where the cultures reached the late logarithmic growth phase, with a final optical density at 600 nm (OD_{600}) of 7 (equivalent to cell density of 7.4 g L^{-1}). That subculture was used to inoculate the biotransformation medium.

In the experiments carried out in RALF PLUS SOLO bioreactor, the subculture cells were used to inoculate 1.7 L of YPD medium in the bioreactor, to give an initial cell concentration of 0.5 g L^{-1}. Cellular growth occurred at 27 °C, 500 rpm and 3 L min^{-1} of aeration rate for 19 h until a final cell density of 30 g L^{-1} and total glucose consumption.

In airlift bioreactor, the subculture cells were used to inoculate 4.5 L of YPD medium in the bioreactor, to give an initial cell concentration of 0.5 g L^{-1}. Cellular growth occurred at 27 °C, 5 L min^{-1} of aeration rate for 48 h until a final cell density of 30 g L^{-1} and total glucose consumption.

3.1.4 BIOTRANSFORMATION MEDIUM

After the biomass production phase, the components of biotransformations medium were directly added, as an emulsion, to the YPD medium containing the cells, in order to start the biotransformation.

According to the results of Gomes et al. (2010), a medium composition of 30 g L^{-1} of castor oil and 3 g L^{-1} of Tween 80 was adopted to use in the experiments. However, in some experiments different oil concentrations were used and therefore the Tween 80 concentration had to be modified, but the ratio between the two compounds was kept constant.

The biotransformation medium composition is presented on Table 3.2.

Table 3.2 – Biotransformatiom medium composition

Compound	Concentration (g L^{-1})
Castor oil	30 or 60
Tween 80	3 or 6
NH_4Cl	2.5
Yeast Nitrogen Base (YNB) with amino acids	6.7

All chemicals were purchased from Sigma-Aldrich (Portugal), except for castor oil, which was purchased from Sociedade Portuense de Drogas, S.A. (Portugal).

3.2 BIOREACTORS

3.2.1 RALF PLUS SOLO BIOREACTOR

A 3.7 L bioreactor (RALF PLUS SOLO, Bioengineering, Switzerland) with 0.31 m height and 0.17 m diameter was used to investigate the influence of experimental conditions and operation mode in γ-decalactone production (Fig. 3.1).

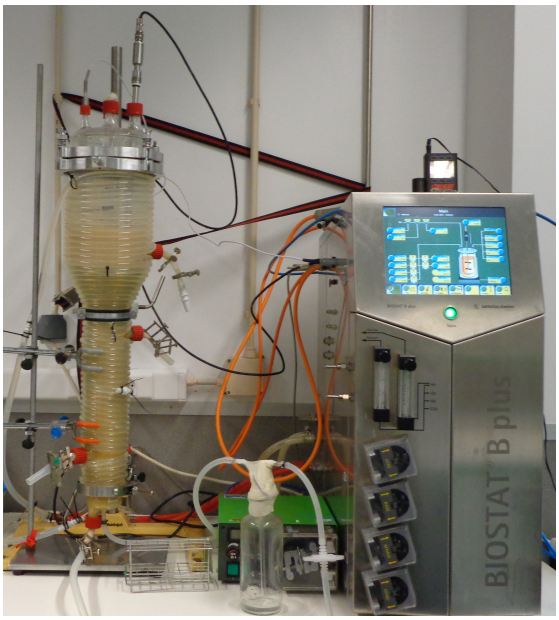
Figure 3.1 - RALF PLUS SOLO bioreactor with biotransformation medium.

Air was supplied with a sparger located at the base of the agitator, with a flow-rate automatically controlled. The medium pH of 6.0 was controlled by addition of 2 N potassium hydroxide or 21 % (v/v) orthophosphoric acid, through Peripex peristaltic pumps (Bioengineering, Switzerland). Dissolved oxygen concentration was measured with a polarographic-membrane probe (InPro 6000, Mettler Toledo, USA) using the BioScadaLab software.

Cellular growth occurred at 27 °C, 500 rpm and 3 L min^{-1} of aeration rate for 19 h until a final cell density of 30 g L^{-1} and total glucose consumption. After the cell growth phase biotransformations were performed.

After defining the optimal operating conditions for γ-decalactone production, the influence of substrate was also analyzed in a step-wise fed-batch strategy, based on two additions of 60 g L^{-1} castor oil.

3.2.2 AIRLIFT BIOREACTOR



An airlift bioreactor was also used to carried out biotransformations experiments (Fig. 3.2).

This bioreactor was constructed in glass with a working volume of 4.5 L and 0.07 m inside diameter. The height of the riser-tube was 0.37 m with an inside diameter of 0.032 m. Air was used as gas stream in the gas-liquid contactor and it was fed at the bottom of the bioreactor using a five holes sparger. The inlet gas flow-rate was measured and controlled with a mass flow controller (Alicat Scientific, USA).

Figure 3.2 - Airlift bioreactor with biotransformation medium.

3.3 OXYGEN MASS TRANSFER DETERMINATION

3.3.1 STATIC GASSING-OUT TECHNIQUE

For the experimental k_La determination in experiments without cells, the static gassing-out technique was used.

Originally proposed by Wise in 1951, this technique allows evaluating the effect of operational variables (like agitation and aeration rates) on the oxygen transfer efficiency.

The oxygen present in the medium is initially removed by gassing the liquid out with

compressed nitrogen, so that the medium is free of oxygen. Aeration is then initiated at a constant air-flow rate until saturation.

The technique is based on the oxygen mass balance equation (Eq. 3.1) which, in the absence of cells and in batch mode, is simplified to the equality between the time variation of the dissolved oxygen concentration (dO/dt) and the oxygen transfer rate from the gas to the liquid.

$$dO/dt = k_L a\,(O^S - O) \qquad \text{(Eq. 3.1)}$$

The integration of this equation gives $k_L a$, which is equal to the symmetrical slope of the line resulting from the graphical representation of the logarithm of $1 - O/O^S$ as function of time (Stanbury and Whitaker, 1984).

Figure 3.3 presents, for illustrative purposes, the evolution of dissolved oxygen concentration in the medium without cells and the graphical representation of the logarithm of $1 - O/O^S$ as function of time.

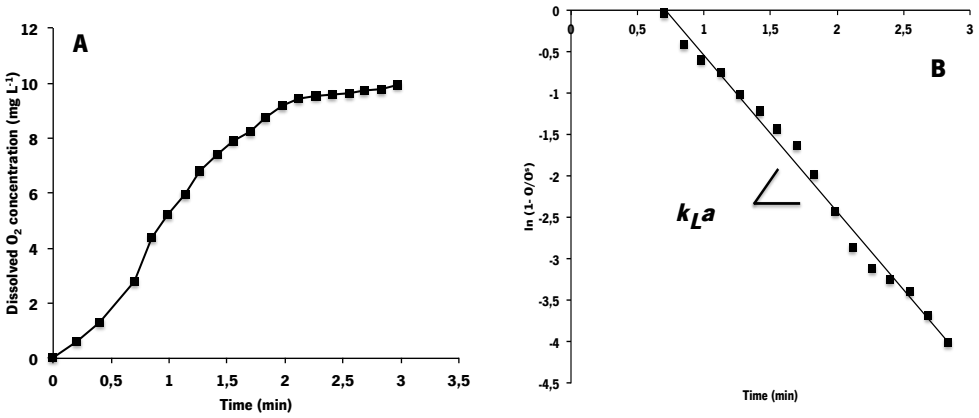


Figure 3. 3 – Time course of dissolved oxygen concentration in the biotransformation medium (A) without cells after re-aeration and (B) logarithmic representation of 1-(O/O^S).

3.3.2 DYNAMIC GASSING-OUT TECHNIQUE

For the $k_L a$ determination during biotransformations, the dynamic gassing-out technique was used. In the presence of active cells and in the absence of aeration, Taguchi and Humphrey (1966) used the respiratory activity of microorganisms to remove oxygen from the medium. This technique has

been performed during biotransformation. The procedure involves two steps: one to stop aeration and another of resumption of aeration in the operating conditions. Thus, in the first step, monitoring the decrease of dissolved oxygen concentration will allow to determine the specific oxygen uptake rate (OUR).

$$dO/dt = - OUR \quad \text{(Eq. 3.2)}$$

Aeration is resumed before reaching the critical dissolved oxygen concentration value (Tribe et al., 1995). After the resumption of aeration (second step), the oxygen mass balance in the liquid phase is expressed by Eq. 3.3.

$$dO/dt = k_L a\,(O^s - O) - OUR \quad \text{(Eq. 3.3)}$$

Considering the pseudo-steady state immediately before the determination, *OUR* can be replaced by Eq. 3.4:

$$k_L a\,(O^s - O_i) = OUR \quad \text{(Eq. 3.4)}$$

Where O_i is the dissolved oxygen concentration in the beginning of the determination. By combination of Eq. 3.3 and Eq. 3.4 and integration of this last equation, results Eq. 3.5:

$$\ln\left(\frac{O_i - O}{O_i - O_o}\right) = - k_L a\,(t - t_0) \quad \text{(Eq. 3.5)}$$

Where O_0 and t_0 are, respectively, the dissolved oxygen concentration and the time, when aeration is resumed.

The graphical representation of the term on the left side of Eq. 3.5, as a function of time, gives a line whose slope is the $- k_L a$ value.

3.4 ANALYTICAL METHODS

3.4.1 CELL CONCENTRATION

3.4.1.1 DRY WEIGHT

Throughout this work, all spectrophotometry measurements were done on a modular absorbance microtiter plate reader (Sunrise, Tecan, Switzerland).

The biomass determination, in growth medium, was determined by dry weight, using a microwave oven (Olsson and Nielsen, 1997). The optical density at 600 nm was converted to g cell dry per liter, using a calibration curve previously obtained.

3.4.1.2 CELL COUNTING

Cell concentration in emulsion medium was determined by direct counting under the microscope, using a Neubauer-improved counting chamber, due to the oil interference in optical density measurements (Paul Marienfeld GmbH & Co, Lauda-Königshofen, Germany) (Mather and Roberts, 1998).

3.4.2 REDUCED SUGARS QUANTIFICATION

Reduced sugars were measured by an adaptation of the dinitrosalicylic acid (DNS) method. In the presence of reducing sugars, 3,5-dinitrosalicylic acid is reduced into 3-amino-5-nitrosalicylic acid, a brownish compound that strongly absorbs light at 540 nm, allowing a quantitative spectrophotometric measurement of the amount of reducing sugars present in a given sample.

The reaction was carried out in wells of 340 μL, adding 25 μL of DNS reagent to 25 μL of sample previously filtered (pore size 0.20 μm, Orange Scientific, Belgium) or distilled water (blank). The microtiter was subsequently placed, with cap, in an oven during 5 minutes, then it was placed on ice and 250 μL of distilled water were immediately added to each well (Gonçalves et al., 2010).

The absorbance values obtained in each assay were converted to a reducing sugar concentration expressed in grams per liter (g L^{-1}), using a calibration curve previously obtained.

3.4.3 LIPASE

Extracellular lipase activity was measured in the samples supernatant according with a previously developed and validated spectrophotometric method for complex systems (Gomes et al., 2011b). The method uses *p*-nitrophenyl- butyrate (*p*-NPB) in sodium acetate buffer 50 mM (pH 5.6) as substrate at 37 °C for 15 min. One unit of activity was defined as the amount of enzyme that produces 1 μmol of *p*-nitrophenol per minute, under assay conditions.

The reaction mixture was composed of 980 μL of substrate (2.63 mM *p*-nitrophenyl butyrate in 50 mM sodium acetate buffer, pH 5.6, with 4% (v/v) Triton X-100) and 20 μL of broth sample. It was incubated for 15 minutes at 37 °C and the reaction was stopped by the addition of 2 mL of acetone. The absorbance was measured at a wavelength of 405 nm.

3.4.4 FATTY ACIDS ANALYSIS

Castor oil consumption was determined by fatty acids methyl esthers (FAME) analysis by Gas Chromatography (GC). Samples (100 mg of semifrozen sample) were placed into a 16 × 125 mm screw-cap Pyrex culture tube to which 1 mL of metanol acidified with H_2SO_4 (10%) and 0.5 mL of the $C_{17:0}$ (Heptadecanoic acid) internal standard (5000 mg of C_{17} mL^{-1} of hexane) were added. The tube was incubated in a 100°C water bath for 2 h with vigorous hand-shaking for 5 s every 30 min to properly permeate, dissolve, and hydrolyze the sample. After cooling below room temperature, 2 mL of hexane was added, and the tube was vortex-mixed for 5 min on a vortex. The tube was centrifuged for 5 min in a centrifuge, and the hexane layer, containing the FAME, was placed into a GC vial.

The FAME phase was analyzed by gas chromatography (Varian 3800 GC) with a TR-WAX capillary column (30 m × 0.32 mm × 0.25 μm) with He as a carrier gas. The temperatures of the split injector and the detector were set to 250 °C and 280 °C, respectively. The oven temperature was programmed to increase from 40°C to 150°C at a rate of 30°C min^{-1} and then to 250°C at a rate of 5°C min^{-1}.

Figure 3.4 presents a typical chromatogram of castor oil FAME's.

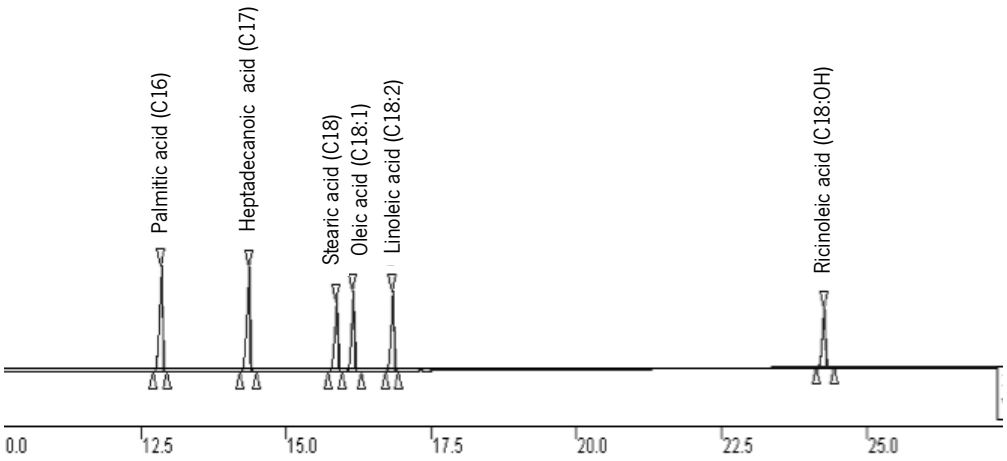


Figure 3.4 - Chromatogram showing separation of fatty acids methyl esters.

3.4.5 LACTONES EXTRACTION AND QUANTIFICATION

For the quantification of lactones, γ-decalactone and 3-hidroxi-γ-decalactone, 2 mL medium samples were removed and their pH was lowered to 2 with HCl to promote the total lactonization of 4-hydroxydecanoic acid. The extraction of lactones was performed with 2 mL of diethyl ether by 60 gentle shakings, after addition of γ-undecalactone as internal standard. After the complete separation of the liquid phases, the ether phase was separated and analyzed by gas chromatography (Varian 3800 instrument, Varian, Inc., USA) with a TR-WAX capillary column (30 m × 0.32 mm × 0.25 μm, Teknokroma, Spain).

Data were analyzed using the acquisition and integration software Star Chromatography Workstation v. 6.30 (Varian, Inc., USA) and to the calibration curves previously obtained for γ-decalactone and γ-undecalactone.

The average of various concentration/area ratios was determined, for both compounds, which allowed determining the response coefficient (K), which is obtained from the ratio between the averages corresponding to γ-decalactone and γ-undecalactone.

The determination of K allows quantifying γ-decalactone using Eq. 3.6.

$$[\gamma\text{-decalactone}] = [\gamma\text{-undecalactone}] \times (1/K) \times (A_{\gamma\text{-decalactone}}/A_{\gamma\text{-undecalactone}}) \quad \text{(Eq. 3.6)}$$

Where [γ-decalactone] represents the concentration of γ-decalactone in mg L^{-1}; [γ-undecalactone] is the concentration of γ-undecalactone in mg L^{-1}; $A_{\gamma\text{-decalactone}}$ refers to the area of γ-decalactone and $A_{\gamma\text{-undecalactone}}$ corresponds to the area of γ-decalactone.

Figure 3.5 presents a typical chromatogram of the different compounds analyzed and the mass spectrum of the compounds.

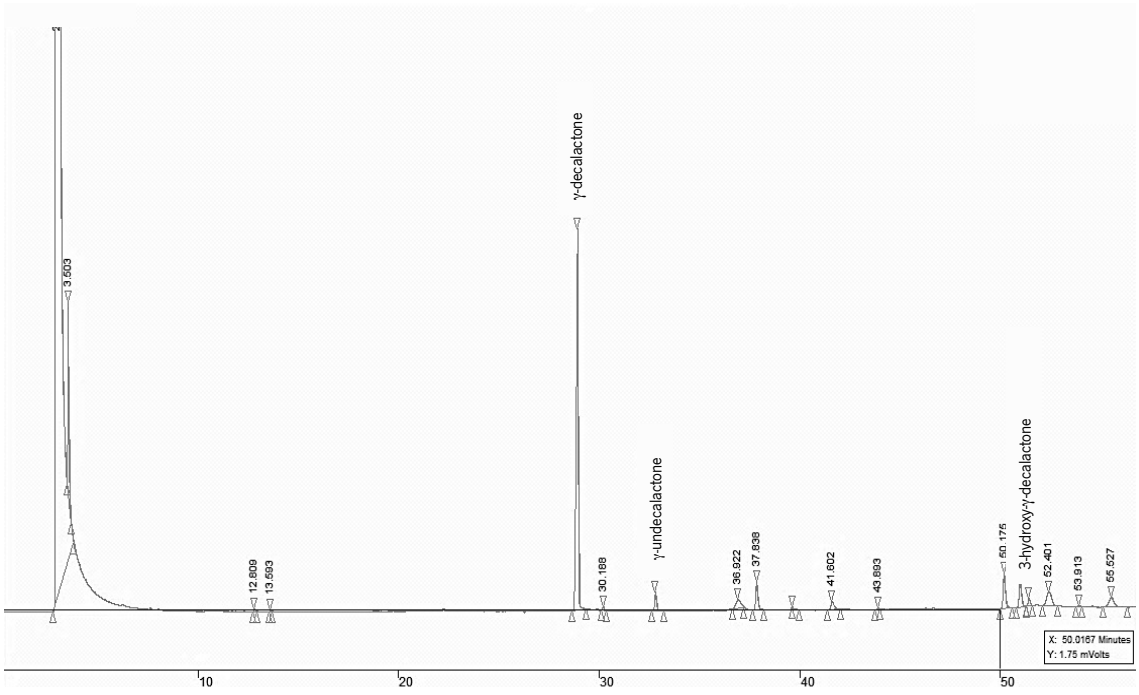
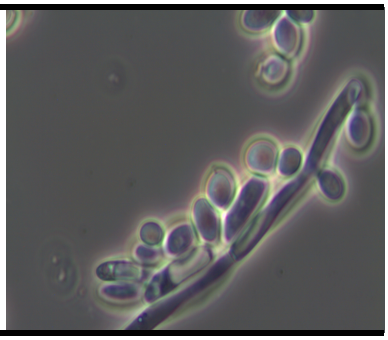


Figure 3.5 - Typical chromatogram with the different compounds analyzed: γ-undecalactone; γ-decalactone and 3-hydroxy-γ-decalactone.

BIOTRANSFORMATION WITH HYDROLYZED CASTOR OIL AND IMMOBILIZED CELLS



Yarrowia lipolytica is one of the yeast species able to carry out the biotransformation of ricinoleic acid into γ-decalactone. Since lipases catalyze the hydrolysis of triacylglycerides into glycerol and free fatty acids, their presence in the oil medium is expected to improve the availability of fatty acid substrates to the microorganism. However, previous work demonstrated that the substrate and lactones produced have a toxic effect to the cells. The immobilization of viable cells is an approach of great interest since, when compared with free cells, immobilized cells exhibit a higher tolerance to toxic compounds and higher productivity. The use of immobilized cells can be seen as a good alternative to protect the cells and improve the aroma production.

As addressed in the previous chapter, most of the processes described in literature present low aroma productivity in most cases probably due to an inefficient hydrolysis of the oil. In this chapter a commercial lipase was tested to hydrolyze castor oil. The castor oil hydrolysis was performed in a previous step to the biotransformation and then aroma production was compared with the obtained when the extracellular lipase was added to medium at the same time as the biotransformation started.

Also, two different supports and culture conditions were investigated in order to achieve a suitable method for *Y. lipolytica* W29 immobilization, and thereafter immobilized cells were used in the biotransformation of ricinoleic acid into γ-decalactone.

The results presented in this chapter were adapted from:

- **Braga, A**.; Gomes, N.; Belo, I. (2012) Lipase induction in *Yarrowia lipolytica* for castor oil hydrolysis and its effect on γ-decalactone production. J. Am. Oil. Chem. Soc. 89(6):1041-1047.

- **Braga, A**.; Gomes, N.; Teixeira, J.A.; Belo, I. (2013) Impact of lipase-mediated hydrolysis of castor oil on γ-decalactone production by *Yarrowia lipolytica*. J. Am. Oil. Chem. Soc. 90(8):1131-1137.

- **Braga, A**.; Belo, I. (2013) Immobilization of *Yarrowia lipolytica* for aroma production from castor oil. Appl. Biochem. Biotechnol. 169(7):2202-2211.

4.1 LIPASE-MEDIATED HYDROLYSIS OF CASTOR OIL

4.1.1 INTRODUCTION

γ-Decalactone is an aromatic compound that can be obtained from the biotransformation of ricinoleic acid by several microorganisms, among which is the aerobe *Y. lipolytica* (Gomes et al., 2010). Castor oil needs to be hydrolyzed in order to release ricinoleic acid to be used in the process.

There are some chemical and physical methods available in the literature to hydrolyze oils. Usually, ricinoleic acid is produced by saponification followed by acidification. Although the reaction conditions are mild (70 – 100 °C), the product obtained has an unacceptable odor and coloration and contains a high quantity of the by-product, Na_2SO_4, which is difficult to remove (Goswami et al., 2009). The high-temperature and high-pressure processes of manufacturing fatty acids are not suitable for castor oil hydrolysis since they lead to the formation of an undesirable product named ricinoleic acid estolide and may cause denaturation of the product (Goswami et al., 2009; Lakshminarayana et al., 1984; Rathod and Pandit, 2009).

However, enzymatic hydrolysis, contrarily to the other techniques, works at moderate temperature, allowing the production of an odorless and light-colored ricinoleic acid (Sharon et al., 1998). Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) are enzymes from the hydrolases family whose main biological function is the catalysis of insoluble triglycerides to generate free fatty acids, mono and diglycerides and also glycerol. Since these enzymes catalyze the hydrolysis of triacyl-glycerides into glycerol, their presence in the medium may improve the availability of fatty acids to the microorganism.

Since most of the processes described in literature revealed low aroma productivity probably due to an inefficient hydrolysis of the oil, the goal of this work was to improve the production of γ-decalactone favoring the hydrolysis of castor oil. For this purpose, the use of Lipozyme TL IM®, a commercial lipase from other microbial sources than *Y. lipolytica*, to hydrolyze castor oil was tested. Then, the biotransformation kinetics using previous hydrolyzed castor oil by Lipozyme TL IM® was compared with the biotransformation were the enzyme was added to the culture medium with non-hydrolyzed castor oil, thus the oil hydrolysis and ricinoleic acid consumption may occur in parallel.

4.1.2 MATERIAL AND METHODS

4.1.2.1 ENZYMATIC HYDROLYSIS OF CASTOR OIL

Commercial lipase, Lipozyme TL IM® from a genetically modified strain of *Aspergillus oryzae,* that expresses a heterologous lipase from *Thermomyces lanuginosus* (enzyme kindly supplied by Novozymes, Bagsvaerd, Denmark), was previously described as a good lipase for the hydrolysis of oils (Kun et al., 2011; Zhang, 2007; Prado and Saldana, 2013) it was tested for castor oil hydrolysis.

The reaction mixture consisted of 30 g L^{-1} castor oil, 3 g L^{-1} Tween 80, 3 mL of 10 mM potassium phosphate buffer (pH 6) and 23.2 U of enzyme. These components were placed in a 25 mL Erlenmeyer flask. The reaction was carried out at 27 °C in an orbital shaker, at 140 rpm, for 50 hours. Samples were removed over time and 4 mL acetone/ethanol (1:1 v/v) was added to stop the reaction. Liberated fatty acids were titrated with 0.1 M alcoholic KOH (ethanol 98 %), using phenolphthalein as an indicator.

The percentage of castor oil hydrolyzed was calculated as indicated by Eq. 4.1:

$$Hydrolysis\ (\%) = \frac{\frac{M \cdot MM \cdot (V - V_B)}{CO \cdot V_S \cdot 1000}}{SV} * 100 \qquad \text{(Eq. 4.1)}$$

Where M (mol L^{-1}) is the molarity of KOH; MM (g mol^{-1}) is the molar mass of KOH; V (mL) is the KOH volume spent in the sample titration; V_B (mL) is the KOH volume spent in the blank titration; CO (g L^{-1}) is the oil concentration in the sample; V_s (mL) is the titrated sample volume; and SV is the saponification value (177 mg KOH g^{-1}, according to the supplier).

4.1.2.2 BIOTRANSFORMATION

For the biotransformation experiments, *Y. lipolytica* W29 was grown in YPD medium as previously described (section 3.1.3) and then, the components of the biotransformation were added to the medium (section 3.1.4).

Experiments were also performed using castor oil enzymatically hydrolyzed (according to the methodology described above) or by adding the commercial lipase Lipozyme TL IM®, to improve the hydrolysis of the oil during the aroma production. In the first case, 10 g of lipase (0.232 U mg^{-1} of enzyme) were added to the flask containing the biotransformation medium (200 mL) and incubated at 140 rpm and 27 °C for 48 h. At this point, the biotransformation medium was inoculated with pre-grown cells (section 3.1.3) that were separated from the growth medium by centrifugation (6000 *g*, 5 min). In the second case, no previous castor oil hydrolysis was performed and the lipase was added to the biotransformation medium with non-hydrolyzed castor oil at the same time as the inoculum cells.

Samples were collected throughout the monitoring period for analysis of lipase activity and γ-decalactone quantification (section 3.4).

4.1.3 RESULTS AND DISCUSSION

4.1.3.1 USE OF A COMERCIAL EXTRACELLULAR LIPASE FOR CASTOR OIL HYDROLYSIS

In biotechnological production of γ-decalactone ricinoleic acid, the main fatty acid of castor oil, is used as aroma precursor. The enzymatic hydrolysis of castor oil is one of the most important steps in γ-decalactone production. In order to increase the castor oil hydrolysis and also aroma production, a commercial lipase, Lipozyme TL IM®, was used.

The most common enzymes described in literature for castor oil hydrolysis are the lipases from castor bean itself (Ory et al., 1960), *Pseudomonas aeruginosa* KKA-5 in presence of metal ions (Sharon et al.,1998), *P. fluorescens* NS2W (Kulkarni, 2002), *A. oryzae* (Kulkarni and Pandit, 2005), *C. rugosa*, *P. cepacia*, and *G. candidum* (Foglia et al., 2000). However all of those enzymes are soluble and can not be reused, that is a disadvantage for it use at industrial level.

Lipozyme TL IM® is produced by a genetically modified strain of *Aspergillus oryzae* that expresses a heterologous lipase from *T. lanuginosa*, immobilized in a silicate via ionic adsorption (Prathumpai et al., 2004; Christensen et al., 2003). This enzyme has selected for castor oil hydrolysis since it is an immobilized lipase, enhancing the operational lifetime and stability of biocatalysts and

facilitates the recovery, reuse and continuous operation. Also, it is largely used in the hydrolysis of different oils and fats to produce free fatty acids, presenting a higher conversion to fatty acids when compared with other lipases (Fernandez-Lafuente, 2010).

The enzymatic hydrolysis of oils may be influenced by several external factors, among which pH and temperature. Since the biotransformation process was performed at 27 °C and pH 6, this temperature was tested for castor oil hydrolysis (Fig. 4.1).

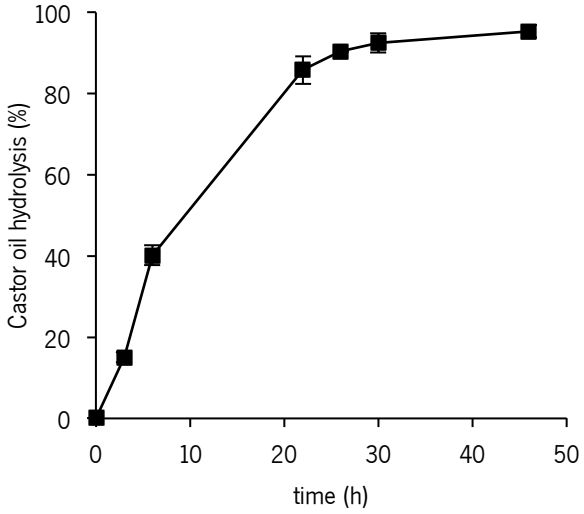


Figure 4.1 - Castor oil hydrolysis with Lipozyme TL IM® at 27 °C and pH 6. Data are presented as the mean and standard deviation of two independent experiments.

The results obtained showed that during the first 8 hours the hydrolysis increased with time and after that reached a plateu, when maximum hydrolysis was reached. As the reaction rate depends on the number of molecules of enzyme bound to substrate molecules, in the initial part of the reaction, there are more interactions between oil and enzyme and consequently the hydrolysis rate is faster. However, as the reaction time proceeds, higher amounts of fatty acids resulting from the oil hydrolysis accumulate and consequently the substrate concentration decreases, also leading to a decrease in castor oil hydrolysis rate (Puthli et al., 2006). Figure 4.1 shows that Lipozyme TL IM® led to a hydrolysis percentage of 95.4 % after 48 h.

4.1.3.2 BIOTRANSFORMATION

Two different aroma production strategies from castor oil were investigated. The first methodology consisted in performing a previous hydrolysis of the oil with the exogenous lipase, Lipozyme TL IM®, before starting the biotransformation; The second strategy consisted in the addition of the same lipase to the inoculated biotransformation medium, with non previous hydrolyzed castor oil.

In all experiments, the γ-decalactone concentration increased up to a maximum and after that, a decrease in the aroma concentration was observed, until complete disappearance of the compound (Fig. 4.2). This decrease in γ-decalactone concentration was due to the fact that this metabolite is consumed by yeasts as a carbon source (Gomes et al., 2010). In the experiments were Lipozyme TL IM® was present, the production of γ-decalactone started to be detected after 48 h of fermentation and the maximal concentration was achieved at 140 h of culture. In the control experiment, the aroma production was detected later (approximately at 100 h), and the maximal concentration was obtained at 185 h. Thus, with the addition of extracellular lipase in the medium it is possible to save around 52 h in the overall process.

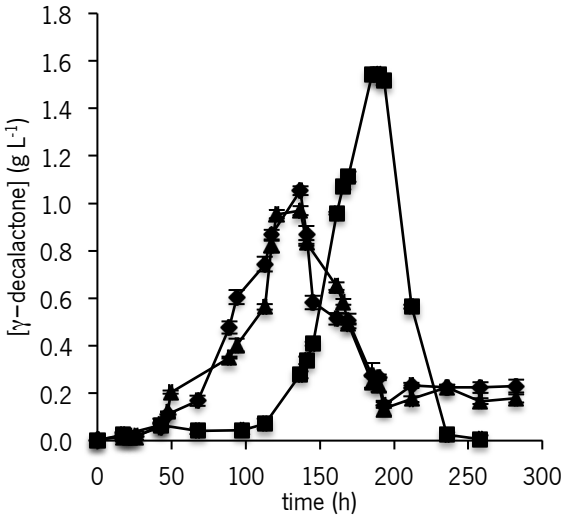


Figure 4.2 - Time course of γ-decalactone accumulation in biotransformation as a result of different production strategies: (♦) with Lipozyme TL IM® added at the beginning of biotransformation; (▲) with castor oil hydrolysis by Lipozyme TL IM® before the biotransformation beginning; (■) without Lipozyme TL IM® addition (control). Data are presented as the mean and standard deviation of two independent experiments.

The existence of a lag phase in γ-decalactone production can be attributed to the time necessary for the synthesis of lipases by the wild type *Y. lipolytica* W29 strain. This lipase are involved in the hydrolysis of castor oil, enabling the availability of ricinoleic acid (which is the substrate for the aroma synthesis) to the cells.

In fact, as observed in Figure 4.3, in the experiment without the addition of extracellular enzyme (Fig. 4.2), a low level of lipase production was observed during the first hours and the peak of lipolytic activity was achieved after the maximum accumulation of γ-decalactone occurred, meaning that the lipase is not expressed right in the beginning of biotransformation and at adequate levels to increase oil hydrolysis and ricinoleic acid liberation.

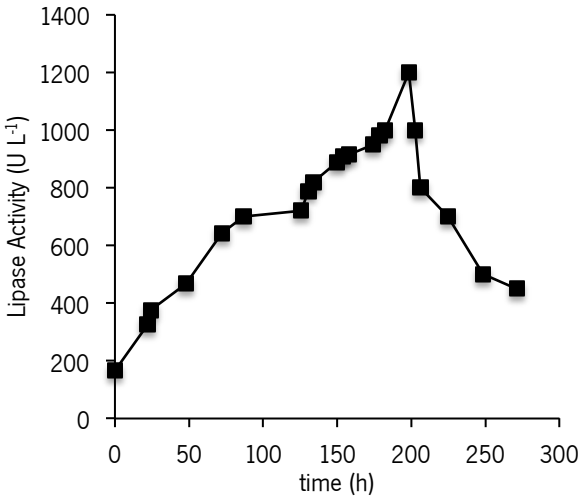


Figure 4.3 – Lipase activity profile in biotransformation media for the experiment without extracellular lipase adittion.

The addition Lipozyme TL IM® decreases significantly the lag phase, since the oil is earlier hydrolyzed into ricinoleic acid. Farbood et al. (1985) also described γ-decalactone production comprising enzymatically hydrolyzing castor oil by an exogenous lipase as an alternative methodology for increasing substrate availability to the cells. In the production of γ-decalactone by *Geotrichum* species, Neto et al. (2004) observed that the addition of ricinoleic acid or hydrolyzed castor bean oil to the culture medium enhanced γ-decalactone production.

However, although the maximum concentration of lactone was reached earlier in the experiments with Lipozyme TL IM®, the accumulated concentration was lower (between 1.2 – 1.0 g L-

^{1}, at 140 h) than that obtained in the control experiments (1.5 g L^{-1}, at 185 h). The lower concentrations of aroma detected in these conditions may be justified by a greater release of castor oil fatty acids, particularly ricinoleic acid (about 90%), linoleic acid (4.2%), oleic acid (3%), stearic acid (1%) and palmitic acid (1%), which can have a toxic effect in yeasts, leading to a lower γ-decalactone production (Waché et al., 2001). When an exogenous lipase is used with the microorganism in the biotransformation, the formation of the enzymatic hydrolysate may be controlled by limiting the amount of lipase used in the process, avoiding toxicity resulting from the presence of excessive amounts of hydrolysate (Farbood et al., 1985). Gatfield (1988) observed that the degradation of ricinoleic acid can lead to the accumulation of other lactones besides γ-decalactone, such as 3-hydroxy-γ-decalactone, dec-2-enolide and dec-3-enolide, which may also contribute to the low yield of aroma.

The productivities in γ-decalactone (calculated in the biotransformation period) obtained in each of the three media tested, medium without lipase addition, with the lipase and previous castor oil hydrolysis and without this preliminary step were 0.083 ± 0.012 g L^{-1} h^{-1}, 0.077 ± 0.012 g L^{-1} h^{-1} and 0.079 ± 0.012 g L^{-1} h^{-1}, respectively. These values are not statistically different which indicates that with the action of Lipozyme TL IM® it is possible to produce γ-decalactone in less time keeping identical values of aroma productivity. In spite of this fact, as no improvement in overall productivity was obtained and the final aroma concentrations were lower, it can be concluded that there is no need of an external lipase in the biotransformation medium for free cells of *Y. lipolytica* W29.

4.1.4 CONCLUSIONS

In order to assess the importance of lipase in the production of γ-decalactone from castor oil, that would enable the faster availability of ricinoleic acid to the cells, a commercial lipase was used for castor oil hydrolysis. Lipozyme TL IM®, at pH 6 and 27 °C, revealed to be an efficient lipase to hydrolyze castor oil, leading to 95.4 % after 48 h.

After investigated the castor oil hydrolysis by Lipozyme TL IM®, the impact of using this enzyme in biotransformation were evaluated and compared with experiment performed without extracellular lipase. As result, the process was faster when Lipozyme TL IM® were present, however γ-decalactone concentrations were lower, nevertheless resulting productivities were similar to those without using lipase.

Following experiments were made with immobilized *Y. lipolytica* W29, in order to protect cells from the toxicity of ricinoleic acid and the lactone. Hydrolyzed castor oil by Lypozyme TL IM® as well as the addition of this enzyme in the biotransformation of non-hydrolyzed castor oil was used, since no aroma production was detected in experiments without extracellular enzyme and immobilized cells (data not show).

Most of the works in the literature that describe γ-decalactone production with immobilized cells use ricinoleic acid as substrate (Lee et al., 1998; Lee et al., 1999). In fact, castor oil is an hydrophobic compound probably only accessible to the external layer of immobilized cells. The extracellular enzyme lead to an increase of ricinoleic acid availability to the cells and reduced the lag phase for γ-decalactone production.

4.2 *YARROWIA LIPOLYTICA* IMMOBILIZATION

4.2.1 INTRODUCTION

Most processes described in the literature report low γ-decalactone concentrations rarely reaching values over 9 to 11 g L^{-1} of fermentation broth. *Yarrowia lipolytica* can use γ-decalactone as a carbon source (Pagot et al., 1998), resulting in its complete depletion from the medium after some hours of batch operation. On the other hand, Waché et al. (2003) observed that lactones have a toxic effect against the producing cells, which results in cell growth inhibition and limitation of the production rate. The decline of cell viability has been shown to be associated with an increase of lactone concentration in culture media during biotransformation (Lee et al., 1998). Also, Lin et al. (1996) observed that the addition of ricinoleic acid to the medium increased γ-decalactone production, but resulted in a significant decrease in the population of living cells with a rate decrease being proportional to the increase of ricinoleic acid concentration.

Thus, an alternative technique should be considered to overcome this problem and improve the aroma production. One approach is the immobilization of viable cells to use in the

biotransformation process since they exhibit a higher tolerance to aroma and ricinoleic acid, that becoming toxic in higher concentrations, resulting in higher productivities than with free cells.

Thus, with the aim of improving the productivity of the aroma process different supports for immobilization of *Y. lipolytica* cells were studied. Different supports and culture conditions were tested in order to achieve a suitable method for cell immobilization. After selecting the best conditions for cell immobilization, free and immobilized cells were used in the biotransformation of ricinoleic acid (presented as castor oil) into γ-decalactone.

4.2.2 MATERIAL AND METHODS

4.2.2.1 CELL IMMOBILIZATION BY ADSORPTION

Methyl polymethacrylate cylinders (C-PMMA) and DupUM® [a thermoplastic support, developed at University of Minho, Portugal (Matos et al., 2011)] were used as support materials for the cells immobilization.

Yeast cells were pre-grown as described in 3.1.2. The influence of different factors (time, cells concentration, pH, total area of support) in the immobilization of *Y. lipolytica* cells by adsorption was studied. After the growth phase, the yeast cells were separated from the growth medium by centrifugation (6000 rpm, 5 min) and transferred to the adsorption media (sodium chloride 10 mM) (Rochex et al., 2004). Different experiments were performed, as described below (Table 4.1), in order to study the influence of different factors in cell adhesion. Cell suspension was kept in contact with the supports during 48 h in an orbital shaker at 140 rpm and 27 °C.

Immobilized cells concentration was achieved by the difference in the absorbance between the adsorption medium in the absence of the support and in its presence.

Table 4.1 - Descriptions of different experiments performed for cell immobilization by adsorption, using two different supports of different materials and shape (schematically shown)

Support	Specific Surface Area ($m^2\ m^{-3}$)	Medium pH	Initial cell concentration (10^7 cell ml^{-1})	$[Cell]_{initial}$ / Support $Area_{total}$ (10^9 cell m^2)	Medium Volume (mL)
 C-PMMA	0. 95	7 9	39.7 ± 0.1	5.2 3.5	50
 DupUM®	407	9	7.86 ± 0.4 39.7 ± 0.5 59.7± 0.4	0.59 7.4 110	90

The influence of the hydrophobicity of cells in the adsorption process was evaluated by the Microbial Adhesion to Hydrocarbons (MATH) assay. Cells, during the adsorption time, were harvested and washed twice with phosphate buffer (0.1 M, pH 7), centrifuged (3000 rpm, 5 min) and re-suspended in the same buffer to an optical density at 600 nm (OD 600) of approximately 0.7 (A_0). In a glass tube, one part of this suspension was mixed with six parts of hexadecane. The tube was gently inverted ten times and left until the separation of the two phases. Then, 2 mL of the aqueous phase were removed and the OD 600 (A) was measured (Aguedo et al., 2003). The results are given as percentage of adhesion (Eq. 4.2):

$$\%\ adhesion = 100 - (100 - \frac{A}{A_0}) \qquad \text{(Eq. 4.2)}$$

4.2.2.2 SUPPORT CHARACTERIZATION

Static contact angle measurements were obtained using OCA 20 from Dataphysics to determine the support hydrophobicity. Water contact angle measurements were performed at room temperature using the sessile drop method (Kwok and Neumann, 1999). The reported angles consist of an average of seven independent measures.

Micrographs of the biocatalysts, after cell adsorption (dried for 24 h at 60 ˚C), were obtained by Scanning Electron Microscopy (SEM) using a Leica Cambridge S360 microscope. To be examined, samples were fixed on a specimen holder with aluminum tape and then sputtered with gold in a sputter coater under high vacuum condition. Each sample was examined at 1000-fold magnification.

4.2.2.3 BIOTRANSFORMATION EXPERIMENTS

In the biotransformation experiments, free and immobilized cells, were used. In the experiments with free cells, cells were pre-grown as described in section 3.1.2.

In order to allow the availability of the substrate (ricinoleic acid) to the cells for γ-decalactone production, hydrolyzed castor oil by Lipozyme TL IM® was used, as well as experiments with the addition of this commercial lipase in the biotransformation medium with non-hydrolyzed castor oil were performed as described in section 4.1.2.1. The yeast cells (free and immobilized) were then transferred to the biotransformation medium.

The reusability of adsorbed cells in DupUM® was assessed using the same immobilized cells in three consecutive batch fermentations. At the end of each 200 h cycle, the spent medium was decanted and immediately replaced with fresh medium for the next cycle. In order to investigate the storage stability of immobilized cells, they were stored at 4°C. Biotransformations were performed after storage for 5, 10, 15 and 30 days.

Samples were collected throughout the monitoring period for analysis of cell and γ-decalactone concentration (section 3.4).

4.2.3 RESULTS AND DISCUSSION

4.2.3.1 CELL IMMOBILIZATION BY ADSORPTION

The adsorption phenomenon is based on electrostatic interactions (van der Walls forces) between the charged support and microbial cells, therefore, their zeta potential plays a significant role in cell-support interactions. Along with the charge on the cell surface, the composition of the cell wall, the support and its properties will also play a relevant role, influencing the cell-support interaction (Kolot, 1981).

For a good immobilization it is important to achieve a high amount of cells adhered to the support surface. So, the influence of different factors (time, cells concentration, pH of the medium and total area of support) in the adsorption of *Y. lipolytica* cells to different supports was studied.

The pH of the adhesion medium is an important factor in the adsorption of cells to surfaces. Since an extreme pH value (<4 or >9) inhibits microbial growth and biosynthesis of extracellular polymers necessary for biofilm formation (Czaczyk and Myszka, 2007), the pH values studied were 7 (neutral), and 9. Figure 4.4 shows that pH was an important variable in this study, since it was observed a decrease in the number of cells adhered, for the higher amount of support area used, when the pH of the medium changed from 9 to 7. This effect was not observed when the support area decreases.

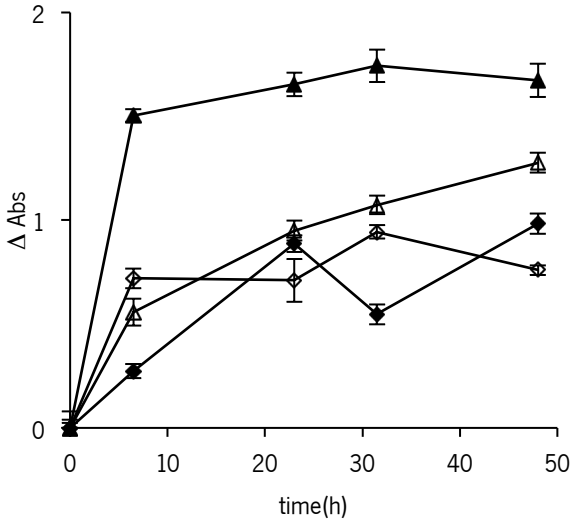


Figure 4.4 - Influence of the contact time, pH and total support area in the adhesion of *Y. lipolytica* onto PMMA cylinders: (▲) pH 9 and 5.34 m^2 support, (◇) pH 7 and 3.56 m^2 support, (△) pH 7 and 5.34 m^2 support, (◆) pH 9 and 3.56 m^2 support. Data are presented as the mean and standard deviation of two independent experiments.

Several procedures of cell adsorption based on pH dependence are reported in the literature (Klein and Ziehr, 1990). Mafu et al. (2011) studied the adhesion of different bacteria to hydrophobic and hydrophilic surfaces, in cultures at different pH (6, 7 and 8) and observed that this variable influenced the adhesion of some bacteria in the material surface.

The net charge of the yeast surface, which could be positive or negative, also depends on pH. For *Y. lipolytica* W29, Aguedo et al. (2005b) reported that the isoelectric point occurs at pH 2.5; and that below this value the net surface charge will be positive and above it will be negative. At the pH values investigated in this work (7 and 9), the surface of the cells is negatively charged and as the pH of the medium increases, the negative charge density of the yeast surface increases due to higher deprotonation of carboxyl, phosphate and amine groups. This effect results in a decrease electrostatic repulsion between the yeast cells and the support, which in turn, has a positive charge, favoring the adhesion phenomenon (Liu, 1995). At pH 7, the repulsive forces due to the net negative charge, start to have a lower impact on the adhesion process, resulting in a decreased number of adhered cells.

The results presented also show that the number of adhered cells was dependent on the contact time between the support and the cells. The adhesion of yeast cells increased linearly with time for 10 h and after that, in general, the number of adhered yeast cells reached a saturation value. Time

course led to an increase in the number of cells collisions with the support surface, and hence, an increase in opportunities for attachment (Fletchet, 1977).

Also, it is possible to observe that the adhesion process was influenced by the total support area. A greater adhesion was obtained in the experiments performed with higher surface area (5.34 m^2). In these conditions, more surface area was available for cell attachment and free cells remained in the medium were able to adhere to the support. In these conditions, the adhesion phenomenon was controlled by the surface of the support.

To confirm the adhesion of cells onto PMMA surface, SEM observations were carried out and micrographs of the support surface and *Y. lipolytica* cells adhered onto the support are shown in Figure 4.5. Cells were attached as single cells.

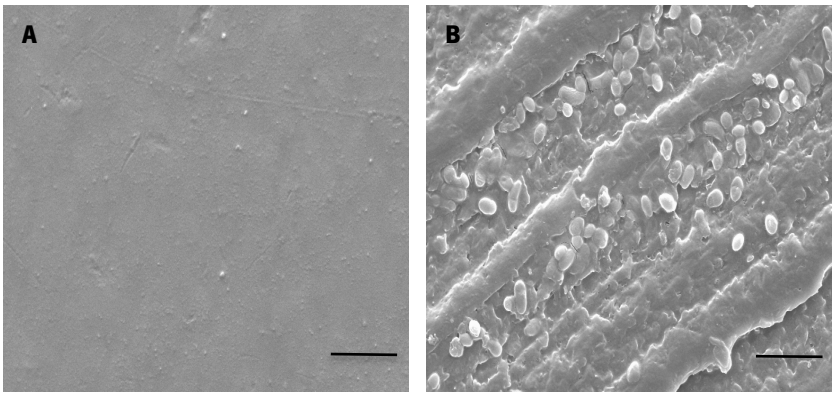


Figure 4.5 - Micrographs by scanning electron microscopy of the PMMA cylinders used for cells immobilization. (A) Support surface before immobilization and (B) after the cells immobilization. The scale bar represents 20 µm.

The main advantage of immobilized systems is to obtain high cell density, thus preventing washout in continuos operation. In order to increase the surface area for cell adhesion, more experiments were performed using DupUM® as support for cell immobilization since this presents a design with high specific area for cell adhesion. The experiments were carried out at pH 9 and 140 rpm (best conditions previously described for cells adhesion on PMMA) varing cell concentration.

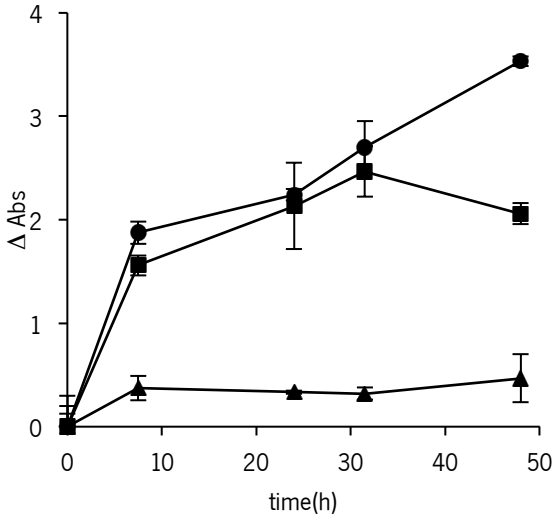


Figure 4.6 - Effect of initial cell concentration in adhesion of *Y. lipolytica* into DupUM®: (▲) $7.7x10^7$ cells mL^{-1}, (■) $3.97x10^8$ cells mL^{-1}, (●) $5.97x10^8$ cells mL^{-1}. Data are presented as the mean and standard deviation of two independent experiments.

Figure 4.6 shows a dependence of cells adhesion to the support upon the initial cell concentration. Works described in literature demonstrate that the dependence of the number of adherent yeast cells upon the initial biomass concentration has a linear behavior (Liu, 1995; Fletcher, 1977; Bellon-Fontaine and Cerf, 1991; Bryers and Characklis, 1981, Rochex et al., 2004). The correlation between cell concentration and the number of adherent cells was analogous to an adsorption isotherm that represents the number of molecules adsorbed per surface unit as a function of concentration. When the initial biomass concentration is lower than the critical concentration, the adhesion may be controlled by the transport rate of the suspended cells from the liquid phase to the support surface. However, once this critical concentration is exceeded, the available adhesion surface of the support probably becomes the adhesion-limiting factor rather than the external mass transport. In addition, it has been reported that cells adhesion is strongly dependent on the available surface for cell adhesion (Rochex et al., 2004; Ohgaki et al., 1978). Also, an increase in concentration or time, led to an increase in the number of yeast collisions with the surface, and hence, an increase in opportunities for attachment (Bellon-Fontaine and Cerf, 1991; Fletcher, 1977). Thus with a 0.7-log and 0.9-log increase in the initial cell concentration, a 4.6-fold and 5.4-fold increase in the cells adhesion was achieved, respectively. Since this support presents a high surface area, the increase of the initial cell concentration results in an increase of cellular adhesion since there are still available free areas on

the support surface. Nevertheless, for cell concentrations of $3.97x10^8$ cell mL^{-1} and $5.97x10^8$ cell mL^{-1}, a similar amount of adhered cells was obtained, since the surface for cell adhesion was completely cover with cells and has no free surface for cells adhesion. Also, the adsorption profiles in these conditions were very similar indicating that the surface of the support becomes an adhesion-limiting factor.

Moreover, an increase of 3.3-fold in the number of adhered cells per surface area was achieved in these experiments when compared with the assays performed with PMMA cylinders (for the best results obtained in each experiment).

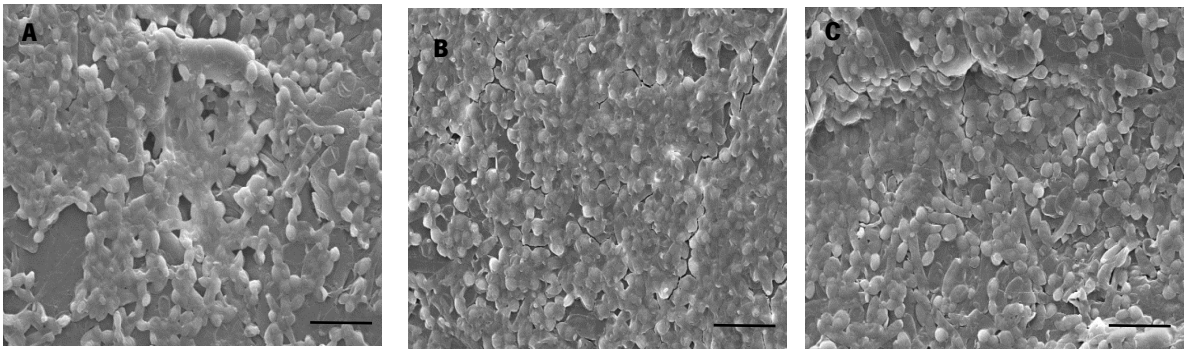


Figure 4.7 - Scanning electron micrographs of *Y. lipolytica* cells adhering to DupUM®. Initial cells concentration (A) $7.7x10^7$ cells mL^{-1}, (B) $3.97x10^8$ cells mL^{-1}, (C) $5.97x10^8$ cells mL^{-1}. The scale bar represents 20 µm.

The microscopic observation of the support surface revealed that the highest amount of adhered cells was obtained with an initial cell concentration of $5.97x10^8$ cells mL^{-1} (Fig. 4.7B). The yeast cells covered the support surface in a thick layer, allowing a greater immobilization with this support. The high specific contact area achieved with the particular shape of the DupUM® support (Table 4.1) enabled a greater amount of cells adsorption compared with PMMA cylinders. This phenomenon has also been reported in other immobilization studies (Brányik et al., 2004; Kosaric and Blaszczyk, 1990; Yu et al., 2010).

In order to better understand the phenomenon of cell adhesion, the relative surface hydrophobicity of the cells was evaluated at different stages of the adhesion process. Several methods have been described in the literature (binding to hydrocarbons, salt aggregation test, adhesion to hydrophobic solid surfaces) (Doyle and Rosenberg, 1990), but the microbial adhesion to hydrocarbons

(MATH) assay is the most practical, since it is easier and less time consuming than other ones (Capizzi and Schwartzbrod, 2001).

In the MATH test 33.6 % ± 0.3 % of cells, from the overall samples, adhered to hexadecane, after the change of the pH of the medium to 9 (results represent an average of three independent experiments ± standard error). These results indicate that around 30 % of the cells present in the medium have a hydrophobic surface and 70 % are hydrophilic.

The value of the water contact angle can give preliminary information on the hydrophobicity of the support. For both supports investigated, the contact angle between water and support was > 50 ° and < 80 °, indicating that the support surface was hydrophilic (Zgura et al., 2010). The results obtained showed that this characteristic of the support facilitated the adhesion of *Y. lipolytica* cells that are majority hydrophilic as shown by MATH test.

4.2.3.2 BIOTRANSFORMATION

After selecting the best conditions for cell immobilization, biotransformations were carried out with immobilized cells in both supports and with free cells. In order to increase the substrate availability and, as previously described, no aroma production was detected in experiments with immobilized cells without addition of extracellular enzyme (data not show), experiments were performed with castor oil previously hydrolyzed by Lipozyme TL IM® and the addition of the same lipase to the medium, with non-hydrolyzed castor oil (Figure 4.8).

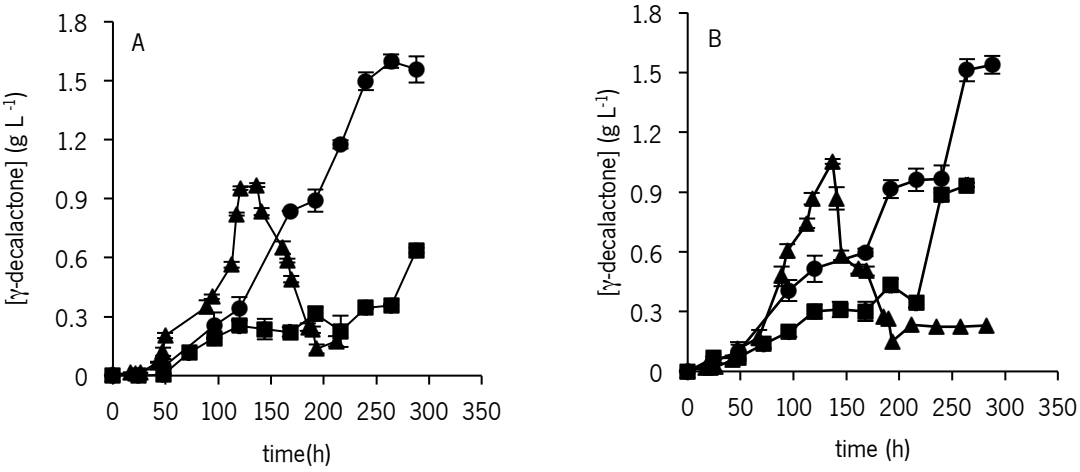


Figure 4.8 - γ-Decalactone production with (A) with previous castor oil hydrolysis and (B) without previous castor oil: (▲) free and immobilized cells: (■) PMMA cylinders, (●) DupUM®. Data are presented as the mean and standard deviation of two independent experiments.

The maximum aroma concentration of 1.6 ± 0.3 g L^{-1} was obtained with cells adsorbed on DupUM® after 264 h, for the two biotransformation strategies studied. In the experiments with free cells, a concentration of around 1.0 ± 0.7 g L^{-1} γ-decalactone was obtained in 136 h. However, the maximum aroma concentration obtained in these conditions is lower than other reported values (Schrader et al., 2004) since these studies were performed with improved strains.

In the experiment with free cells, the γ-decalactone concentration increases up to a maximum value after which decreases, until complete disappearance from the medium. This decrease in γ-decalactone concentration is due to the fact that this metabolite is consumed by yeasts as a carbon source, or is resumed and used for the production of other products of the β-oxidation pathway (Gomes et al., 2010). Nevertheless, this behavior was not observed with immobilized cells and the aroma concentration was maintained in the biotransformation medium during the whole experiment. This may be related with the fact that in the experiments with immobilized cells, γ-decalactone is not so accessible to immobilized cells, thus its reconsumption is slower than with free cells. Moreover, immobilized cells are protected from the inhibitory effect of the γ-decalactone concentration in the bulk medium. This is a great advantage of using immobilized cells when compared with free cells, since, in these conditions, the aroma is not consumed and the subsequent recovery and purification process is easier. However, some diffusional limitations may occur for the immobilized systems and lower production rates were obtained in these conditions (0.33 g L^{-1} h^{-1}, 0.65 g L^{-1} h^{-1} and 0.86 g L^{-1} h^{-1}, for immobilized cells in PMMA cylinders, DupUM® and free cells, respectively).

When comparing the productivities of γ-decalactone (calculated in the biotransformation period) obtained in each of the two media tested (Table 4.2), no significant differences were observed, which indicates that the substrate is equaly accessible for cells in both systems, e.g., the use of the extracellular lipase in the biotransformation medium gives identical results as the use of a previous castor oil hydrolysis by the enzyme, but the adittion of enzyme at the beginning of biotransformation was preferable since a gain of 48 h is obtained, once no pre-hydrolysis step was performed.

Table 4.2 – Maximum productivity of γ-decalactone, with free and immobilized cells, obtained for different medium tested

Medium	Support	Productivity (g L^{-1} h^{-1})
Previous castor oil hydrolysis	PMMA cylinders	0.02 ± 0.01
	Dup UM®	0.05 ± 0.01
	Free cells	0.07 ± 0.01
Without previous castor oil hydrolysis	PMMA cylinders	0.03 ± 0.01
	Dup UM®	0.06 ± 0.01
	Free cells	0.08 ± 0.01

Thus, the best approach to increase γ-decalactone production, is to adsorb *Y. lipolytica* cells on DupUM® (Fig. 4.8 B) and add the extracellular lipase Lipozyme TL IM® to the biotransformation medium without previous hydrolysis, since a greater amount of γ-decalactone was obtained and the aroma remained in the medium. This strategy is preferable to the one that uses hydrolyzed castor oil, since the same aroma production was obtained but in this case, the previously oil hydrolysis step is not necessary.

The highest productivity of the process was obtained with free cells (0.08 ± 0.01 g L^{-1} h^{-1}), but the maximum aroma concentration was obtained with adsorbed cells in DupUM®, that have an important impact on the further extraction and purification of the aroma.

4.2.3.3 STORAGE STABILITY AND REUSE OF IMMOBILIZED CELLS IN DupUM®

In order to be economically interesting, the immobilized systems must be stable and reusable. In order to investigate the storage stability of immobilized cells in DupUM® for γ–decalactone production, immobilized cells were stored at 4°C for 5, 10, 15 and 30 days and thereafter biotransformations experiments were performed (Figure 4.9).

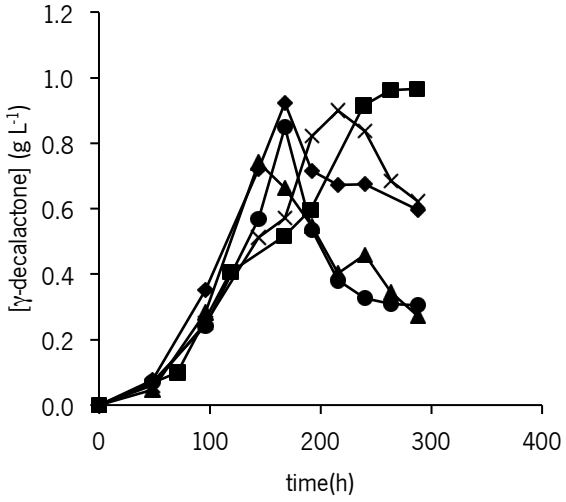


Figure 4.9 - Comparison of storage stability of immobilized cells in DupUM® at 4ºC for: (■) 0d; (▲) 5d; (●) 10 d; (◆) 15d; (x) 30d.

As shown in Figure 4.9, with the extension of storage time, immobilized cells hold a stable γ-decalactona production after being stored for 30 d at 4 °C, with an aroma production of around 1 g L^{-1}, while a decrease in aroma concentration was observed only in the experiments with stored cells. In this case a switch to other pathways in the β-oxydation cycle may occur, decreasing γ-decalactone concentration.

Another important advantage of cell immobilization is the reuse of cells to improve productivity (Groboillot et al., 1994; Saswathi et al., 1995). Paik and Glatz (1994) observed that immobilized *Propionibacterium acidipropionici* in calcium alginate beads could be reused for consecutive fermentation of propionic acid. Also, Lee et al. (1999a) during production of γ-decalactone by immobilized *Sporidiobolus salmonicolor* within calcium alginate beads observed that immobilized cells can be reuse for 13 consecutive 4-day batch fermentations and the aroma production was about 58.4 % of the one obtained in the first cycle.

Furthermore, DupUM® immobilized cells were tested in three consecutive biotransformations to determine if there was deactivation of cells after repeated use. Maximum production of γ-decalactone of ca. 1 g L^{-1} was noted in the first cycle, while in the other consecutive cycles, γ-decalactone production ranged from 0.8 to 1 g L^{-1}. In the 3^{th} cycle, the last cycle tested, γ-decalactone production was ca. 80 % of the one obtained in the first cycle. This indicated that immobilized cells could be reused for at least three cycles. This results were similar to the ones reported by Paik and

Glatz (1994) who used immobilized *P. acidipropionici* for ten consecutive fermentations for propionic acid production and indicated that the concentration of propionic acid produced in the 10th cycle was about 50 % of the one obtained in the 1st cycle. Stability of imobilzed cells was proven by the results obtained by Wang et al. (2007) who studied biodegradation of phenol by immobilized *Acinetobacter sp.* which where stable after reuse for 50 times. Furthermore, neither free cells were observed in biotransformation medium and the medium remained clear after the final cycle, which demonstrating that DupUM® retained a high mechanical strength and nor contamination with other organisms was detected. These results suggest that DupUM® immobilized *Y. lipolytica* cells can be used for several consecutive fermentations with adequate and acceptable performance.

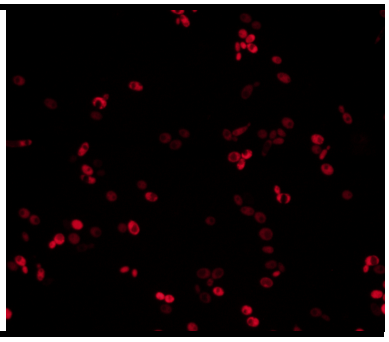
4.2.4 CONCLUSIONS

The best approach to increase γ-decalactone production, is to adsorb *Y. lipolytica* cells on DupUM® and add the extracellular lipase Lipozyme TL IM® to the biotransformation medium, since a greater amount of γ-decalactone was obtained and the aroma remained in the medium.

Furthermore, immobilized cells hold a stable γ-decalactone production after being stored for 30 d at 4°C. Also after reuse in three consecutive biotransformations, γ-decalactone production was ca. 80 % of that in the first cycle, indicating that immobilized cells could be reused for at least three cycles.

This is a very promising result for γ-decalactone production, with potential to be used at industrial level since the use of immobilized cells system will facilitate the conversion of a batch process into a continuous mode keeping high cell density and will allow easier recovery of metabolic products.

BIOTRANSFORMATION AT BIOREACTOR LEVEL



Yarrowia lipolytica is able to grow on hydrophobic substrates such as oils, n-alkanes, fats and fatty acids, for which it has specific metabolic pathways. The main metabolic pathway of fatty acids is through β-oxidation peroxisomal. The pathway from ricinoleic acid to γ–decalactone involves four β-oxidation cycles yielding 4-hydroxy-decanoyl-CoA, which is then, cyclized to γ-decalactone. As an intervening factor in the metabolic pathway involved in this biotransformation, oxygen plays an important role in the reactions of production and consumption of the aroma. For this reason it is imperative to define the most appropriate conditions of oxygenation for lactones industrial production.

In this chapter different strategies were studied in order to improve γ-decalactone production by *Y. lipolytica* W29 on a medium containing castor oil, as ricinoleic acid source, focusing on the influence of oxygen transfer rate on γ–decalactone production in stirred tank and airlift bioreactors and comparing the aroma production in both systems. The influence of mechanical and pneumatic agitation in cell morphology was also investigated using image analysis.

The results presented in this chapter were adapted from:

- **Braga, A.**; Belo, I. (2014) Production of γ-decalactone by *Y. lipolytica*: insights into experimental conditions and operation mode optimization. J. Chem. Technol. Biot. DOI: 10.1002/jctb.4349.

- **Braga, A**.; Mesquita D.P.; Amaral A.L.; Ferreira E.C. ; Belo I. (2014) Comparing γ-decalactone production in an airlift bioreactor and a STR: influence of oxygen in aroma production and morphological characterization using image analysis. Biochem. Eng. J. (submitted).

5.1 STR AND AIRLIFT BIOREACTORS: EFFECT OF OXYGENATION

5.1.1 INTRODUCTION

In aerobic cultures using non-hydrosoluble substrates, such as the use of castor oil for the biotransformation of ricinoleic acid into γ-decalactone by *Y. lipolytica*, the selection of bioreactor type is particularly important. Mass transfer phenomena in this complex system include the oxygen mass transfer from the gas to the liquid phases, between the liquid hydrophobic and aqueous phases, and from the liquid phases to the cells. In classical stirred tank bioreactors high shearing rates have been used to break down air bubbles and substrate globules, and thus increase the interfacial area for mass transfer (Dumont and Delmas, 2003; Yoshida et al., 1970; Rols and Goma, 1989). Nevertheless, such agitation conditions tend to separate cells adhered to substrate globules which, in turn, can be prevented using aeration as the driving force promoting agitation (the principle behind airlift reactors). Thus, other designs have been proposed and investigated since decades, among which airlift reactors (Chisti, 1989).

Airlift bioreactors are pneumatically agitated and often employed in bioprocesses where gas-liquid transfer is important. Some attractive features of airlift bioreactors are the low power consumption, simplicity in construction with no moving parts, high mass and heat transfer rates and uniform shear distribution (Chisti and Moo-Young, 1989; Merchuck et al., 1994).

The characterization of the oxygen mass transfer ability of each type of bioreactor is very important for its applicability in aerobic processes, being described by the volumetric oxygen mass transfer coefficient (k_La), a crucial parameter of bioprocesses scale-up. k_La depends on a large number of parameters: physical properties of gas and liquid phases, operating conditions and the bioreactor geometric design (Garcia-Ochoa and Gomez, 2009; Suresh et al., 2009).

Several empirical correlations have been proposed to estimate k_La in mechanical agitated bioreactors, being the most well-known the following:

$$k_La = \alpha\,(P_g/V)^{\delta}\,(v_s)^{\gamma} \qquad \text{(Eq.5.1)}$$

where P_g represents the power input to the aerated bioreactor, V the bioreactor working volume, υ_s the superficial gas velocity and α, δ and γ are dimensionless constants.

As in stirred bioreactors, similar empirical correlations have been proposed to estimate k_La values in airlift reactors and being expressed generally in the form:

$$k_La = C \times \upsilon_s^{\alpha} \quad \text{(Eq.5.2)}$$

where C is a constant, υ_s the superficial gas velocity and α is a dimensionless constant.

Yarrowia lipolytica is a dimorphic organism capable of growing in two distinct morphological forms, usually as single oval cells or as filamentous hyphae, reversibly. It is believed that the dimorphism of this yeast, as well as other species, is a mechanism to overcome stress conditions (Kawasse et al., 2003). Thus, the yeast morphology is strongly influenced by the growth conditions (aeration, carbon and nitrogen sources, pH, dissolved oxygen concentration in the medium, etc) (Cruz et al., 2000). The system motion, ensured by mechanical mixing or induced circulation, may have a significant effect on cellular activities. In fact, the living cells used in several bioprocesses are suspended in the culture broth, where their walls can be damaged by a series of turbulent fluid dynamic conditions produced by the stirring device, among which is shear stress. The existence of short and large yeast cells, alongside filaments with high ramification frequency, under steady stirring conditions, has been reported in the literature, implying that the morphology of a given mycelium seems to be consistently influenced by the mixing conditions (Buckland et al., 1988). In industrial fermentations, the cells morphology can significantly affect the rheological properties of the medium, the oxygen transfer rate, the nutrients consumption rate, and the progress of the cellular metabolic mechanisms (Walker, 1998). Although yeast cells may still grow in adverse conditions, morphological changes can be observed. Microscopy techniques, combined with image analysis, are regarded as quite useful tools for yeasts morphological characterization towards the enlightenment of the environmental conditions dependency.

Nowadays, the automatic analysis of numerical images captured by digital cameras allows for quickly extracting of quantitative information (Pons and Vivier, 1998). Thus, microscopy image analysis techniques have gained, during the last years, an unquestionable role in several fields of research regarding yeasts characterization (Coelho et al., 2004, 2007). Quantitative image analysis (QIA) procedures can provide valuable information about the biological processes, and allow for a monitoring

tool to decide if a given action should be carried out in the system. However, to the author' knowledge, QIA application has never been considered for studying the morphological changes in *Y. lipolytica,* regarding the use of different reactors configuration to favour γ-decalactone production.

Thus, the influence of mixing conditions and oxygen transfer rate on γ-decalactone production by *Y. lipolytica* in biphasic culture medium with castor oil as substrate, was evaluated. The influence of mechanical and pneumatic agitation in the yeast cell morphology was also investigated using QIA.

5.1.2 MATERIAL AND METHODS

5.1.2.1 OXYGEN MASS TRANSFERENCE AND k_La MODELING

For the k_La determination the static gassing-out technique was used, as previously described in section 3.3.1.

P_g and υ_s in equation 5.1 and 5.2 were calculated with the help of following equations that converting aeration rate to real F_g. To calculate the power input to the aerated system (P_g), the Reynolds number (N_{Re}) is determined by equation 5.3 and the power number (N_p) by equation 5.4,

$$N_{Re} = (D_i^2 \times N \times \rho) / \upsilon \qquad \text{(Eq. 5.3)}$$

$$N_p = P_g / (\rho \times N^3 \times D_i^5) \qquad \text{(Eq. 5.4)}$$

where ρ represents the liquid density, N the agitation rate, υ the liquid viscosity and D_i the impeller diameter.

According to Cheremisinoff and Gupta (1983), if the flow regime inside the system is turbulent ($19070<N_{Re}<38141$), N_p is not a function of N_{Re} when the vessel is fully baffled. Consequently, P_g without aeration (P_g') can be determined by equation 5.5,

$$P_g' = K_T \times D_i^5 \times N^3 \times \rho \qquad \text{(Eq. 5.5)}$$

where K_T is a constant dependent on the impeller used. Finally, to determine P_g in an aerated

system, equation 5.6 can be used,

$$P_g = c \times ((P_g' \times N \times D_i^3)/(F_g^{0.56}))^{0.45} \quad \text{(Eq. 5.6)}$$

where c is a constant dependent on the impeller and F_g is the volumetric gas flow rate.

The dimensionless parameters α, δ, γ were estimated by minimizing the sum of least squares of the differences between the experimental and modeled value of k_La, using the Solver tool of Microsoft Excel 2011 software.

For the k_La determination in biotransformation experiments, the dymanic gassing-out technique was used (section 3.3.2).

5.1.2.2 BIOTRANSFORMATION IN STR AND AIRLIFT BIOREACTORS

After the growth phase, the biotransformations take place in both bioreactors. The components of the biotransformation medium were added to the YPD medium containing the cells, in order to start the biotransformation phase. Biotransformations were carried out in a 3.7 L operating volume STR bioreactor (section 3.2.1) with a flow-rate of 1.7 L min^{-1} and 5.1 L min^{-1} and a stirring rate of 400 rpm, 500 rpm and 650 rpm. Also, some biotransformations experiments were performed in a 4.5 L operating volume airlift bioreactor (section 3.2.2) with air flow-rates of 6 L min^{-1}, 7.5 L min^{-1} and 9 L min^{-1}.

Samples were collected throughout the monitoring period for aroma compounds quantification (section 3.4).

The lipidic material was also visualized and analyzed by Nile blue A staining. The methodology was adapted from Ostle and Holt (1982) and further reported in Mesquita et al. (2013), by incubating 1 mL of sample (supernatant) with 1 drop of Nile blue A for 10 min. Oil droplets were visualized using epifluorescence microscopy by means of an Olympus BX51 (Olympus, Tokyo, Japan) fluorescence microscope at 400× total magnification.

5.1.2.3 IMAGE ANALYSIS

SAMPLES PREPARATION

During the monitoring period, samples were also collected for morphological characterization using QIA in bright-field microscopy. 1 mL of each sample was collected and centrifuged at 5000 rpm for 5 min. The supernatant was discarded and the pellet cells were washed and re-suspended with distilled water and centrifuged using the same conditions. This procedure was repeated three times in order to remove all lipidic material. The pellet cells were then re-suspended in 1 mL of distilled water. Afterwards, samples were combined with the same volume of safranin O (0.25% v/v, Panreac) and then visualized in bright-field microscopy.

BRIGHT-FIELD IMAGE ACQUISITION

The safranin stained yeast cells were observed by means of an Olympus BX51 (Olympus, Tokyo, Japan) optical microscope, at 400× magnification, coupled with an Olympus DP25 (Olympus, Tokyo, Japan) digital camera. Image acquisition was further performed according to Mesquita et al. (2011).

BRIGHT-FIELD IMAGE ANALYSIS

The image processing and analysis was based on the identification, quantification, and morphological characterization of yeast cells using Matlab 7.8.0 (The Mathworks, Natick, MA). A detailed description of the developed image processing and analysis program is presented below.

The first step of the QIA procedure was based on the enhancement of the color images by background removal. In this stage the original image was first divided by a previous acquired background image to minimize background light differences. Afterwards, the resulting RGB image was split into the three composing channels (red, green and blue), and the segmentation took place using the image of the minima of the three channels. Next, objects connected to the image border as well as

small debris were suppressed. Furthermore, the application of hole-fill procedure, and distance based morphological operations was further used, to fill object holes and enhance the borders definition. The resulting binary image was further used for characterization, upon the isolation of individual cells. The yeast cells were classified according to their size, based on the equivalent diameter (D_{eq}) in small (D_{eq} < 2 µm), intermediate (2 µm < D_{eq} < 10 µm) and large cells (D_{eq} > 10 µm).

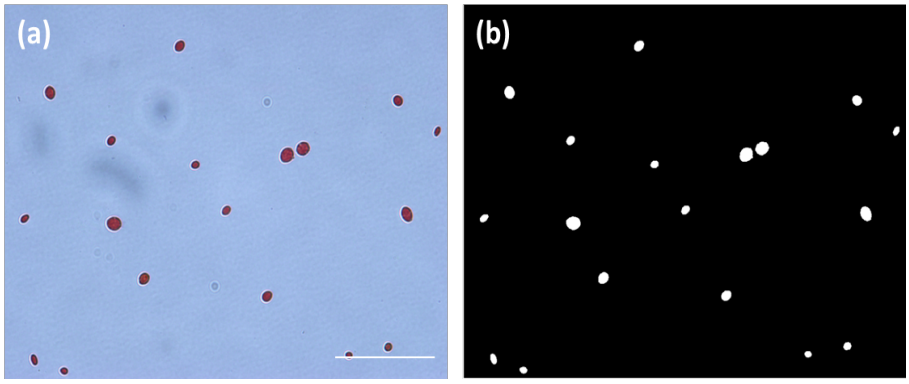


Figure 5.1 - (a) Original image and (b) final binary image. The scale bar represents 50 µm.

5.1.2.4 MORPHOLOGICAL CHARACTERIZATION

Supported on the previous study of Amaral and Ferreira (2005) and Mesquita et al. (2009) several parameters were determined and are described below. The equivalent diameter (D_{eq}) was calculated based on the area determination by the following equation:

$$D_{eq} = 2F_{cal}\sqrt{\frac{Area}{\pi}} \qquad \text{(Eq. 5.7)}$$

where F_{Cal} is the calibration factor (µm.pixel^{-1}).

Other parameters are based on the Feret Diameter which is the maximum distance between two parallel tangents touching opposite borders of the object (Glasbey and Horgan, 1995).

Eccentricity is calculated using the area and the second order moments (M_2) of an object determined by (Glasbey and Horgan, 1995):

$$ECC = \frac{(4\pi)^2(M_{2X}-M_{2Y})^2+4M_{2XY}^2}{Area^2} \quad \text{(Eq. 5.8)}$$

Compactness was calculated using the area and the maximum Feret diameter (F_{Max}) of an object based on the following equation (Russ, 1995):

$$Comp = \frac{\sqrt{\frac{4}{\pi}}\times Area}{F_{Max}} \quad \text{(Eq. 5.9)}$$

Robustness was given by the following equation (Pons et al., 1997):

$$Robus = \frac{2er_{obj}}{\sqrt{Area}} \quad \text{(Eq. 5.10)}$$

where the er_{obj} is the erosions needed to delete an object.

5.1.3 RESULTS AND DISCUSSION

5.1.3.1 OXYGEN MASS TRANSFER IN AIRLIFT AND STR BIOREACTORS

Mass transfer modeling in biphasic systems has been conducted by several authors (Gomes et al., 2007; Nielson et al., 2003). Biphasic systems have been used to improve mass transfer from the gas to the aqueous phase, where the second liquid phase (organic) is usually an inert compound, like perfluorocarbons and silicone oils, among others (Dumont and Delmas, 2003). On the other hand, several biotechnological processes have been developed in biphasic systems formed by an oil-in-water emulsion, in which the oil is the substrate to be degraded by microorganisms.

This is the case of γ-decalactone production by *Y. lipolytica* where the culture medium is an oil-in-water emulsion composed of castor oil, or its derivatives, stabilized by Tween-80. In two-liquid phase systems, with the organic phase dispersed in the aqueous phase, the effect of the organic compound on k_La can be taken into account (Gomes et al., 2007) that was not applied in this work since constant oil concentration was used.

Experimental results of k_La obtained for the different experimental conditions investigated in STR and airlift bioreactors are depicted in Figures 5.2A and 5.2B, respectively.

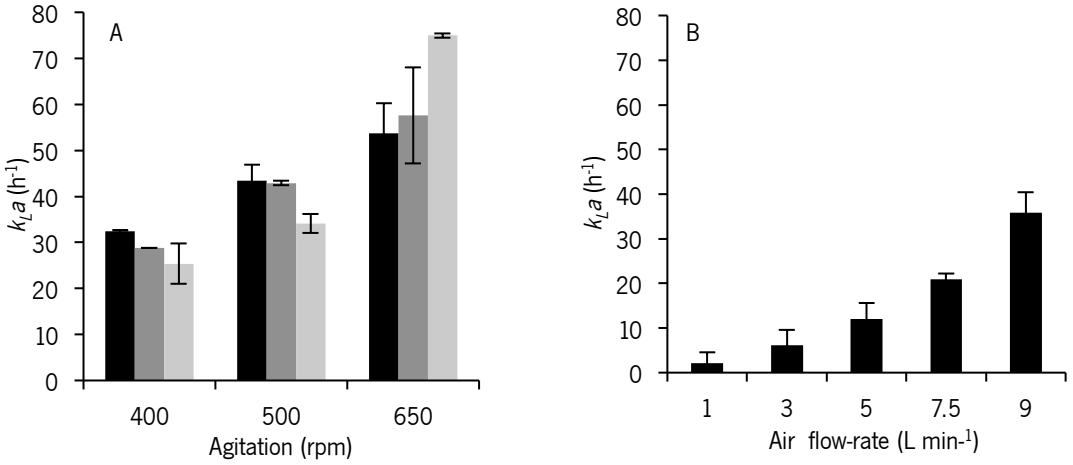


Figure 5.2 - Experimental k_La values at various experimental conditions in (A) STR, air flow-rate: (■) 1.7 L min^{-1}; (■) 3 L min^{-1} (■) 5.1 L min^{-1} and (B) airlift bioreactors. Data are presented as the mean and standard deviation of two independent experiments.

From the results in Figure 5.2A it is possible to observe that for the STR, increasing the agitation from 400 rpm to 650 rpm and aeration rate from 1.7 L min^{-1} to 5.1 L min^{-1} resulted in a 2.3-fold increment of the k_La. It was also found that the agitation was the most important factor affecting k_La, since its increase from 400 rpm to 650 rpm, at constant air flow-rate of 5.1 L min^{-1}, led to a 2.9-fold increase in k_La. This behavior is in agreement with the results of Amaral et al. (2008), Gomes et al. (2007) and Gómez-Diaz et al. (2008) which showed that k_La depends more strongly on agitation than on aeration rates. In the STR, the increase of aeration had no significant effect in k_La values ($p<0.05$) for the lower agitation speeds (see Figure 5.2A). This is due to the fact that increasing mixing induces the fine dispersion of air bubbles in the biotransformation medium, thus resulting in the gas-liquid interfacial area increase. In the airlift bioreactor (Fig. 5.2B), the aeration rate had a strong impact in k_La since an increment of the air flow-rate from 1 L min^{-1} to 9 L min^{-1} resulted in a 16.3-fold increase of the k_La. This observation is in agreement with previous experimental works (Carvalho et al., 2000, Peter et al., 2006; Blažej et al., 2004; Samuel et al., 2005; Zhang et al., 2006; Zhonghuo et al.,

2010). Comparing both reactors, for the tested conditions and similar air flow-rates, higher k_La values were observed for the STR with respect to the airlift bioreactor.

A number of empirical correlations have been proposed to estimate k_La values in different reactor types. Dimensional equations establish relationships between k_La and superficial gas velocity, fluid properties and bioreactor geometry (mainly column height and diameter, and sparger characteristics), although the last ones present relatively little influence. The STR dataset (Fig. 5.2A) was fitted by Equation 5.1 resulting in Equation 5.11 and from the airlift dataset (Fig. 5.2B) was fitted by Equation 5.2 resulting in Equation 5.12, with k_La in h^{-1}, P_g in W, V in m^3 and υ_s in m s^{-1}.

$$k_La = 18\ (P_g/V)^{0.51}\ \upsilon_s^{0.24} \qquad \text{(Eq. 5.11)}$$

$$k_La = 759\ \upsilon_s^{1.85} \qquad \text{(Eq. 5.12)}$$

Figure 5.3 compares the predicted with the experimental k_La values in both bioreactors. For the airlift the predicted k_La was 1.03 ± 0.09 the experimental k_La, and for the STR the predicted k_La was 0.84 ± 0.08 the experimental k_La. Given the obtained regression coefficients (R^2) of 0.86 and 0.98, for STR and airlift respectively, it could be concluded that Equations 5.11 is a worse fitting of data than 5.12.

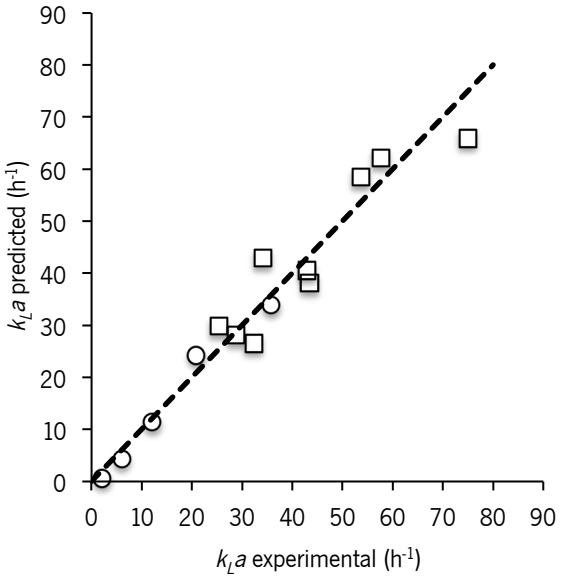


Figure 5.3 - Correlation between the experimental and predicted k_La values in (□) STR and (O) airlift bioreactors.

From Equation 5.11 it can be observed that k_La depends on a power function of 0.51 with the specific power input, (P_g/V), and on a power function of 0.24 with the superficial gas velocity (υ_s). The obtained power coefficients confirm that k_La depends strongly on the specific power input than on the superficial gas velocity, given the lower υ_s power coefficient. According to Kawase and Moo-Young (1987), for aqueous systems, the suggested values for δ (Eq. 5.1) are between 0.37 and 0.80, while, for γ (Eq. 5.1), the values are between 0.4 and 0.84. Comparing the obtained values with the ones previously described in the literature, it was found that δ was within the range specified by Kawase and Moo-Young (1987). However, for γ the found value was lower. It should be kept in mind though, that those studies were performed in water systems and this work described a biphasic system. In fact, Sauid et al. (2013) studied the oxygen mass transfer in a biphasic system with palm oil and observed that the presence of vegetable oil could increase the k_La and, depending on the oil fraction added to the medium, alter the γ exponent between 0.12 and 0.34. These values indicate that the functional dependence of k_La on operating conditions for two-phase systems is slightly different from single-phase systems. Furthermore, it reinforces the k_La dependence on agitation, a very important factor to increase the organic phase dispersion in the aqueous phase, improving gas–liquid mass transfer (Dumont and Delmas, 2003).

According to Bello et al. (1985) and Barboza et al. (2000), for aqueous systems, the suggested values for υ_s exponent in Equation 5.12, ranges between 0.5 and 1.3, that is lower than the 1.85 constant values found for υ_s exponent obtained in the current study.

The expected higher υ_s coefficient value obtained for the airlift, comparing with the STR, shows the stronger effect of the superficial gas velocity on k_La in pneumatic agitated compared to mechanical stirred bioreactors.

5.1.3.2 γ-DECALACTONE PRODUCTION

From the studied set of conditions, biotransformations experiments were performed for selected *OTR* conditions in airlift and STR bioreactors.

Table 5.1 - Volumetric mass transfer coefficient (k_La) obtained for the airlift (under different air flow-rates) and STR (under different air flow-rates and agitation speed) reactors. Data are presented as the mean and standard deviation of two independent experiments

Airlift			STR		
Experimental conditions (air flow-rate L min^{-1})	**k_La (h^{-1})**	**Productivity (g L^{-1} h^{-1})**	**Experimental conditions (Agitation and air flow-rates)**	**k_La (h^{-1})**	**Productivity (g L^{-1} h^{-1})**
6	16 ± 4	0.03 ± 0.04	**400 rpm 1.7 L min^{-1}**	39 ± 3	0.02 ± 0.06
7.5	21± 3	0.04 ± 0.07	**500 rpm 5.1 Lmin^{-1}**	69 ± 3	0.03 ± 0.08
9	35 ± 3	0.07 ± 0.09	**650 rpm 5.1 L min^{-1}**	113 ± 5	0.06 ± 0.01

From Table 5.1 it is clearly observed that k_La has a positive influence on aroma productivity, within each reactor. The increase in γ-decalactone productivity is a consequence of the increase in the production rate and not on the maximum aroma concentration obtained (Fig. 5.4). In fact, Figure 5.4A indicates a maximum γ-decalactone concentration of 2.9 ± 0.1 g L^{-1}, obtained at 104 h with the lower air flow-rate used (6 L min^{-1}) for the biotransformation in the airlift. The same behavior is shown in Figure 5.4B, with the maximum aroma concentration (1.5 ± 0.4 g L^{-1}) obtained for the lower aeration rate tested (1.7 L min^{-1}) in the STR.

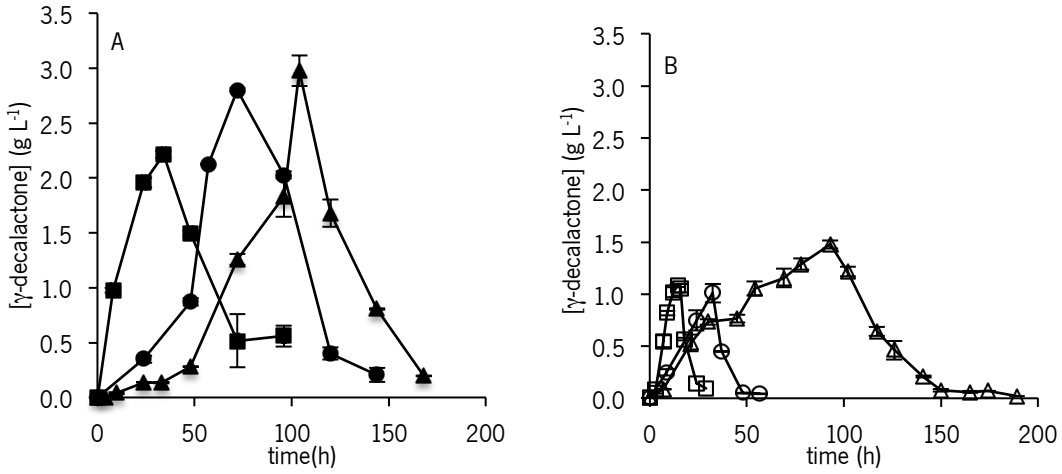


Figure 5.4 - Accumulation of γ-decalactone in the biotransformation medium under different operating conditions in the (A) airlift bioreactor: (▲) 6 L min^{-1}; (●) 7.5 L min^{-1}; (■) 9 L min-^{1} and in (B) STR: (Δ) 400 rpm and 1.7 L min-^{1}; (O) 500 rpm and 5.1 L min^{-1}; (□) 650 rpm and 5.1 L min^{-1}. Data are presented as mean and standard deviation of two independent experiments.

Increasing the oxygen transfer rate reduces the time needed to reach the peak of γ-decalactone production, resulting in higher productivities than the ones obtained using low oxugenation conditions (Table 5.1). This is in agreement with the results obtained by Aguedo et al. (2005a) and García et al. (2007). Both works concluded that low oxygen concentrations in the medium induce the control of the β-oxidation pathway by acyl-CoA oxidase and therefore, an accumulation of γ-decalactone occurs.

Gomes (2011a) studied the γ-decalactone production in airlift bioreactors and further observed that an increase on the oxygen transfer rate led to inversely proportional aroma concentrations, obtaining 1 g L^{-1} aroma with an air flow-rate of 1 L min^{-1}. The higher γ-decalactone concentrations obtained in the present work could be partly attributed to the Perspex airlift reactor used by Gomes (2011a). In fact, the use of Perspex may lead to cell adhesion on the reactor surface, thus lowering the amount of "viable" cells in the biotransformation medium for efficient substrate utilization. Furthermore, when the adhered cells form a biofilm, oxygen transfer may be further reduced within it.

Comparing the results obtained for the two bioreactors, higher aroma concentrations were obtained for the airlift, although needing larger times for maximum γ-decalactone concentration. On the other hand, in the STR for higher oxygen transfer rate, the process was very fast, with maximum aroma production at 12 h, a full 24 h earlier than in the airlift.

3-Hydroxy-γ-decalactone production was also analyzed since, as previously described, the accumulation of this compound can provide useful information concerning the β-oxidation metabolic pathway control. According to Figure 5.5, in general, the production of this lactone was inverse to γ-decalactone: higher oxygen transfer rates led to higher concentrations.

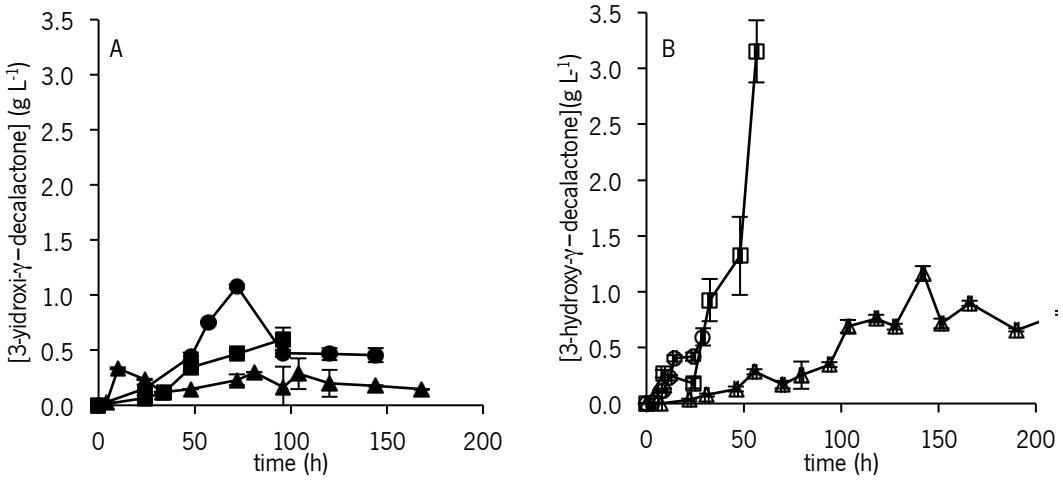


Figure 5.5 - Accumulation of 3-hydroxy-γ-decalactone in the biotransformation medium under different operating conditions in the (A) airlift bioreactor: (▲) 6 L min^{-1}; (●) 7.5 L min^{-1}; (■) 9 L min^{-1} and in (B) STR: (Δ) 400 rpm and 1.7 L min^{-1}; (O) 500 rpm and 5.1 L min^{-1}; (□) 650 rpm and 5.1 L min^{-1}. Data are presented as mean and standard deviation of two independent experiments.

The increase of 3-hydroxy-γ-decalactone concentration in the medium was also dependent on oxygen transfer, in agreement with the results from Aguedo et al. (2005a) who observed that 3-hydroxyacyl-CoA dehydrogenase was induced by high oxygen levels, resulting in an accumulation of this lactone. Furthermore, García et al. (2007) have also reported this compound synthesis for high *OTR* conditions. Higher 3-hydroxy-γ-decalactone concentrations were obtained in the present work, regarding the study of García (2008) on 3-hydroxy-γ-decalactone production in an airlift bioreactor (1.5 g L^{-1} at 0.493 vvm). As above mentioned, the used Perspex based reactor may have induced cells to adhere to the reactor surface, lowering the cells in the biotransformation medium, and possibly forming a biofilm with limited oxygen transfer.

Comparing the results obtained for the two bioreactors it is possible to observe higher hydro-lactone concentrations for the STR.

Despite of similar productivity values in both bioreactors (Table 5.1), although these values were obtained at lower k_La values in airlift bioreactor, the hidden effect of mixture could play an important role and mask the differences observed.

Due to the limited solubility of oil in water, cells growth in a medium containing lipids is influenced by the size of the oil droplets (Aguedo et al., 2003). Thus, the oil droplets size on the emulsion, can strongly affect aroma production. Observations of oil droplets during its biotransformation by *Y. lipolytica* showed a quite dissimilarity between airlift and STR bioreactors (Fig. 5.6). It was found that larger oil droplets were obtained with the lower air flow-rate for the airlift bioreactor (6 L min^{-1}) (Fig. 5.6A) when compared to the STR for all tested conditions (Fig. 5.6D to 5.6F). Furthermore, it was found that the air flow-rate increase in the airlift led to smaller oil droplets (Figure 5.6B and 5.6C), thus increasing mixture. On the other hand, the increase on the aeration rate and agitation speed seemed to have no significant impact on the oil droplets size, leading in all cases to small oil droplets, indicative of a good mixture.

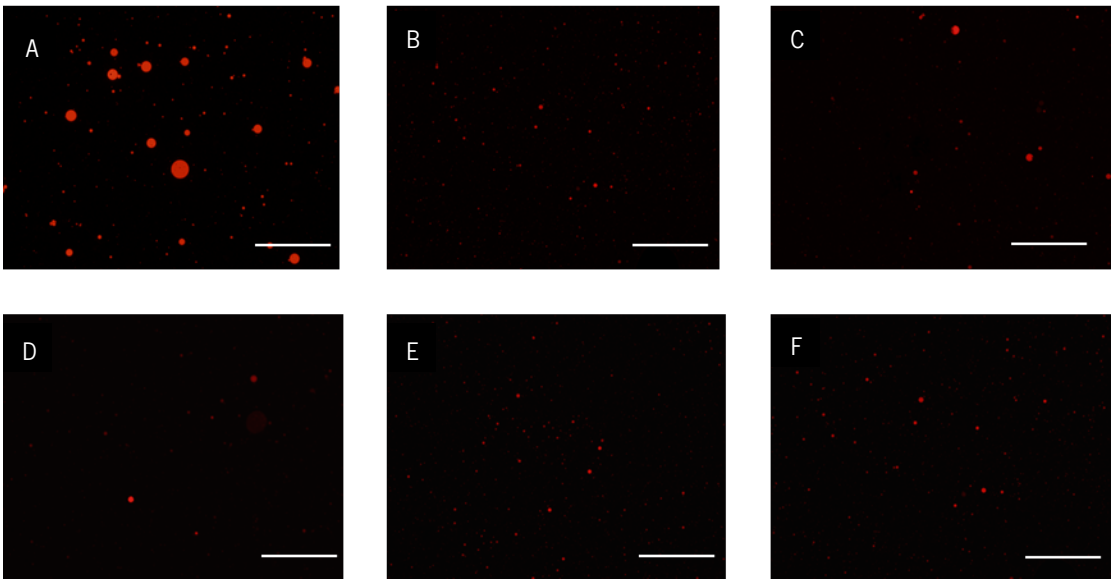


Figure 5.6 - Castor oil droplets stained with Nile blue A, in airlift (A) 6 L min^{-1}; (B) 7.5 L min^{-1}; (C) 9 L min^{-1} and STR reactors (D) 400 rpm and 1.7 L min^{-1}; (E) 500 rpm and 5.1 L min^{-1}; (F) 650 rpm and 5.1 L min^{-1}. The scale bar represents 50 µm.

Previous studies from Aguedo et al. (2003) revealed that the contact between cells and droplets occurs mainly through the adhesion of small-sized droplets on the surface of the yeast. In the present case, after microscopic visual inspection, it was found that smaller castor oil droplets were more favourable to the aroma production. This corroborates the theory that the cells contact with the substrate occurs by the adsorption of small oil droplets in the cell surface.

Another possible explanation for the differences observed in the two systems is the impact of mechanical mixing or induced circulation in the cell morphology. Thus, the study of cell morphological and size changes, through microscopy and image analysis techniques, enables a better understanding of cellular adaptation mechanisms (Szaniszlo, 1985; Vanden Bossche et al, 1993).

In this study, QIA was used to investigate possible morphological changes in *Y. lipolytica* cells caused by the increase of the air flow-rate, and to compare between mechanical and pneumatic mixing. In Figure 5.7 images of *Y. lipolytica* cells in airlift and STR reactors are presented. It is possible to observe that in STR experiments pseudo-hyphae form of the cells increased with respect to the airlift. This result indicates that mechanical agitation may cause increased stress in the cells with respect to pneumatic agitation. These results are in agreement with the work of Kawasse et al. (2003) showing that *Y. lipolytica* under thermal and oxidative stress increased hyphae formation, as a cell mechanism response to stress conditions. Also the effect of low dissolved oxygen was previously reported by Cruz et al. (2000) in dimorphic yeasts as a stress factor causing the conversion from oval cells to hyphae.

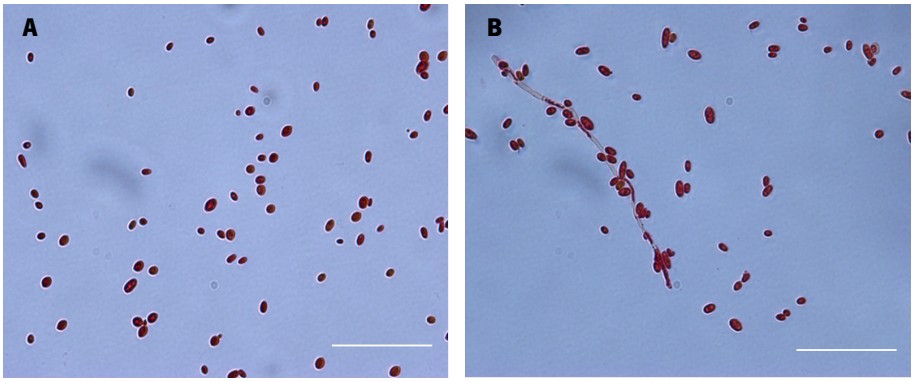


Figure 5.7 - *Yarrowia lipolytica* cells stained with safranin in (A) airlift bioreactor, and (B) STR. The scale bar represents 50 µm.

Taking into account the information provided by image analysis, it is known that the equivalent diameter (D_{eq}) is a size related variable; compactness represents the space fulfilling ability; eccentricity

the elongation of the object, and robustness conjugates the fulfilling ability and the border's roughness (Amaral and Ferreira, 2005). In previous studies, it has been already found the usefulness of evaluating different cell size classes using image analysis information (Coelho et al., 2004, 2007). In the current work, *Y. lipolytica* cells were divided in three classes, with respect to the equivalent diameter: below 2 μm, between 2 and 10 μm, and above 10 μm. The larger size class was considered to represent attached cells; the intermediate size class was composed by normal cells and the smaller size class configured individual bud cells (after the release from the budding cell). The equivalent diameter of each cell class was evaluated to survey the effect of the reactor configuration in the cells size. First, a comparison was performed concerning the D_{eq} behavior for each bioreactor. It was found that the D_{eq} obtained for each size class and for both reactor configurations was kept constant during the monitoring period and in average around 8.1 μm (data not shown). Thus, only average results were further considered since no substantial differences were observed when the three classes were analyzed separately.

An in-depth analysis was then performed regarding the morphological characteristics of the cells. The cells morphological characterization (Fig. 5.8) revealed that when the airlift bioreactor was operated with air flow-rate of 6, 7.5 and 9 L min^{-1}, no significant dissimilarities were obtained, indicating that the aeration provided as the driving force to promote agitation favoured the presence of compact (high robustness values around 0.81), regular (high compactness values around 0.86), and somewhat elongated cells (eccentricity values around 0.68).

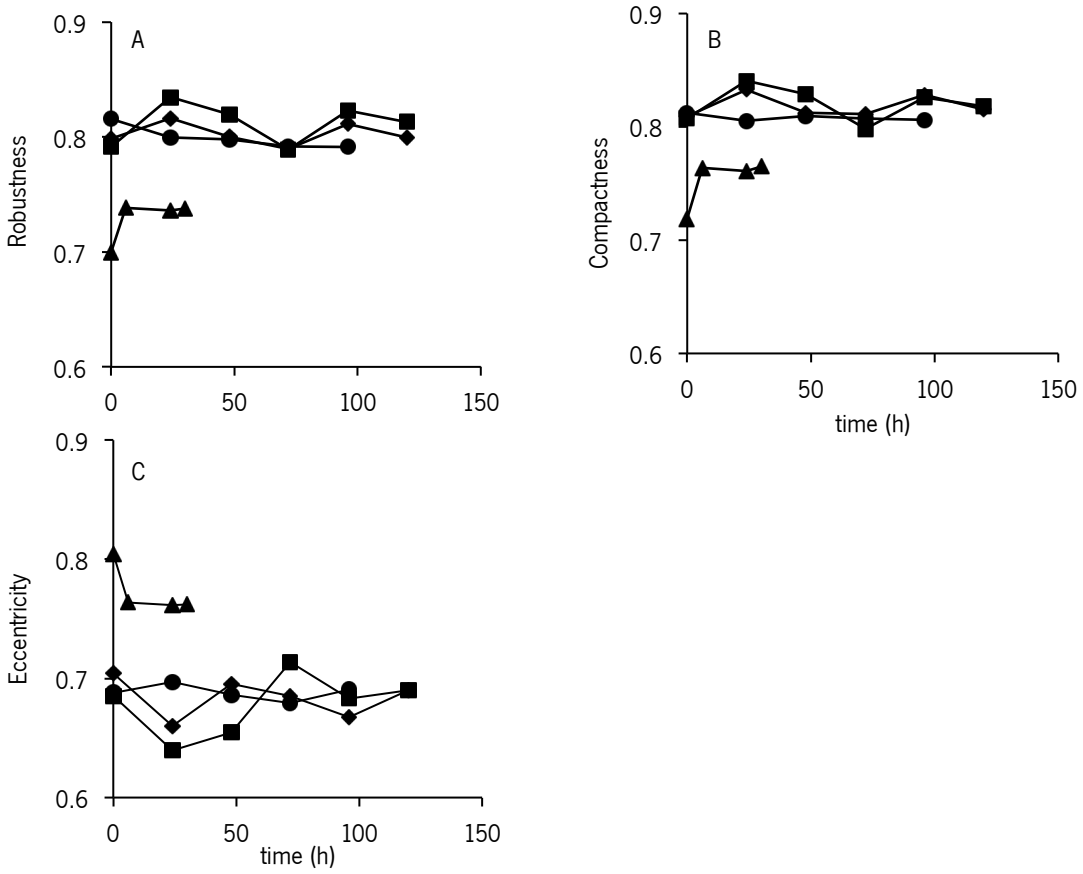


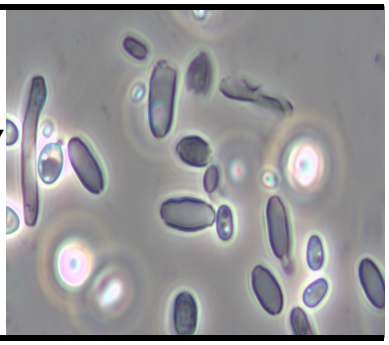
Figure 5.8 - Morphological parameters. (A) Robustness, (B) Compactness, and (C) Eccentricity for the characterization of the airlift (♦) 6 L min^{-1}; (●) 7.5 L min^{-1}; (■) 9 L min^{-1} and (▲) STR reactors.

The analysis of the most significant morphological parameters of the STR (for 650 rpm and 5.1 L min^{-1}), showed a predominance of somewhat less compact (lower values of 0.75 for compactness), more elongated (higher eccentricity values around 0.77), and more irregular cells (lower robustness values around 0.72). This indicated that the cells could also be mechanically more fragile. Thus, it could be established, by the cells morphological characterization based on QIA, that pneumatic agitation should be preferred in the present case and that mechanical agitation caused a fair amount of stress to the cells.

5.1.4 CONCLUSION

γ-Decalactone production from castor oil by *Y. lipolytica* W29 has been studied in an STR and an airlift reactor, taking into account the effect of oxygen transfer rate and bioreactor design. Independently of the bioreactor type, *OTR* increase improved γ-decalactone production rate but decreased the maximum aroma concentration achieved. A maximum γ-decalactone concentration of around 3 g L^{-1} was obtained in the airlift for lower k_La values. Applying QIA techniques for cells morphological characterization it was possible to show that in airlift bioreactor cells were more compact, regular and somewhat elongated contrarily to STR, where cells were less compact, more elongated and irregular. Thus, the use of airlift bioreactors, which do not require mechanical agitation, a major cause of shear stress to the cells, may offer an alternative system for γ-decalactone larger scale production using *Y. lipolytica*.

γ-DECALACTONE PRODUCTION BY *Y. LIPOLYTICA* MUTANTS



γ-Decalactone production from ricinoleic acid biotransformation by *Y. lipolytica* has taken the attention of many authors and developments have been achieved using the wild-type strain W29. However, this strain also degrades the lactone produced due to the high level of acyl-CoA oxidase activity in *Y. lipolytica* W29.

In this chapter, the performance of *Y. lipolytica* strains with modifications in the lipid metabolism at the β-oxidation pathway (acyl-CoA oxidases) and the triglyceride hydrolysis (*Lip2* overexpression) was monitored, using castor oil as substrate.

The results presented in this chapter were adapted from:

- **Braga, A**.; Belo, I. (2014) Production of γ-decalactone by *Y. lipolytica*: insights into experimental conditions and operation mode optimization. J. Chem. Technol. Biot. DOI: 10.1002/JCTB.4349

- **Braga, A**.; Crutz-Le Coq, A.M.; Dulermo, R.; Nicaud, J.M.; Belo, I. (2014) Effect of *POX* genotype and *Lip2p* overexpression on lactone production and reconsumption during biotransformation of castor oil by *Yarrowia lipolytica*. J. Ind. Microbiol. Biot. (submitted).

6.1 BATCH *VERSUS* FED-BATCH CULTURES FOR γ-DECALACTONE PRODUCTION

6.1.1 INTRODUCTION

β-Oxidation pathway is the classical biochemical route involved in fatty acids degradation. It acts on an acyl-CoA molecule and consists of four-step reaction sequence, yielding an acyl-CoA, which has two carbons less and an acetyl-CoA. This sequence is repeated several times until the complete breakdown of the compound.

The commonly accepted pathway from ricinoleic acid to γ-decalactone is presented in Figure 2.2: four β-oxidation cycles occur, yielding 4-hidroxy-decanoyl-CoA, which is then cyclised to γ-decalactone. The yeast *Y. lipolytica* possesses a family of six acyl-CoA oxidases (Aox1 to 6 encoded by *POX1* to *POX6*). The first enzyme of the pathway is generally considered as the limiting step in the catalysis (Groguenin et al. 2004). Nowadays, the main topic in the strategies to improve the γ-decalactone production yields concerns the β-oxidation pathway (Pagot et al., 1998; Waché et al., 1998, 2000b, 2001; Blin-Perrin et al., 2000). Deleting genes coding for short-chain selective acyl-CoA oxidases (Aox3p) enabled to increase the production of γ-decalactone (Waché et al., 2000b) and decrease the one of other lactones (Waché et al., 2001). Following these observations, an attempt to block β–oxidation on short-chains was carried out and it resulted in a 10-fold increase in the yields (Waché et al., 2002; Groguenin et al., 2004). In addition, modification of β-oxidation fluxes at the multifunctional enzyme can trigger the switch between the production of γ-decalactone and the production of hydroxy-related lactones. The yeast *Y. lipolytica* seems to have a low activity at the multifunctional enzyme level and this result in the accumulation of the three hydroxy-related lactones.

Besides these studies at molecular level, strategies to improve aroma production also addressed the culture conditions. The effect of medium composition on γ-decalactone production has been studied. Braga et al. (2013) investigated the influence of different castor oil concentrations (10 g L^{-1}, 30 g L^{-1} and 50 g L^{-1}) on γ-decalactone production by *Y. lipolytica* W29 in flask experiments, and concluded that the best substrate concentration was 30 g L^{-1} of castor oil, obtaining an aroma

production of 1.8 g L^{-1}. Fed-batch operation allows higher cell density than batch mode and is often applied to obtain high yields and productivities, by controlling the nutrient feeding. Gomes et al. (2012) obtained a γ-decalactone productivity of 0.043 g L^{-1} h^{-1} for a step-wise fed-batch operation applied to *Y. lipolytica* cultures, when 30 g L^{-1} methyl ricinoleate was fed twice to the bioreactor.

However, still low concentration of γ-decalactone have been obtained mainly due the degradation of newly synthesized lactone and the partial use of ricinoleic acid or intermediate at the C_{10} level, which is simultaneously the precursor for other γ-lactones (Rabenhorst and Gatfield, 2002).

Therefore, the γ-decalactone production using castor oil as ricinoleic acid source by different genotypes *Y. lipolytica* strains and their response to increased substrate concentration was characterized.

6.1.2 MATERIAL AND METHODS

Yarrowia lipolytica cells (Table 3.1) were grown as described in section 3.1.3. After the cell growth phase, the biotransformations take place in STR bioreactor. The components of the biotransformation medium were added to the YPD medium containing the cells, in order to start the biotransformation phase. Biotransformations were carried out in a 3.7 L operating volume STR bioreactor (section 3.2.1) with a air flow-rate of 5.1 L min^{-1} and a stirring rate of 650 rpm, with two different castor oil concentrations, 30 g L^{-1} and the double of this concentration, 60 g L^{-1}.

The influence of substrate concentration was also analyzed in a step-wise fed-batch strategy, based on two additions of 60 g L^{-1} castor oil after a batch phase with same substrate concentration.

Samples were collected throughout the monitoring period for analysis of extracellular lipase activity, substrate and lactones quantification (section 3.4).

6.1.3 RESULTS AND DISCUSSION

6.1.3.1 EFFECT OF CASTOR OIL CONCENTRATION

The influence of castor oil concentrations on γ-decalactone production was investigated, using the *Y. lipolytica* WT strain W29 and two modified strains. MTLY40-2P is affected in its β-oxidation pathway and is expected to produce higher levels of γ-decalactone due to severely decreased short-chain fatty acid degradation and enhanced long-chain fatty-acid β-oxidation (Waché et al., 2002). In Chapter 4 it was pointed out the importance of extracellular lipase addition for the fast release of ricinoleic acid from castor oil. Thus JMY3010 strain, in which an additional copy of *LIP2* gene, coding for the main lipase expressed by *Y. lipolytica* under the pTEF promoter, was used .

Figure 6.1 shows the γ-decalactone production (filled symbols) in batch cultures of the three strains with 30 g L^{-1} and 60 g L^{-1} of castor oil. Briefly, it was observed that for all strains the increase of castor oil concentration from 30 g L^{-1} to 60 g L^{-1} increased the aroma production. This increase was higher for WT and MTLY40-2P strains were the highest value of γ-decalactone was reached (c.a 4.1 g L-1 ± 0.4). An et al. (2013) also studied the influence of different substrate concentrations for the production of γ-dodecalactone by permeabilized *Waltomyces lipofer* cells. They observed that increasing the substrate concentration resulted in proportional increases in the production of γ-dodecalactone. However, the production reached a plateau for substrate concentrations higher than 60 g L^{-1}. Our results contrasted with those reported by Alchihab et al. (2010) whom observed that γ-decalactone concentration obtained with the strain *Rhodotorula aurantiaca* was did not increase with the concentration of castor oil added.

However, the two mutant strains produced higher aroma concentration for 30 g L^{-1} castor oil than the WT.

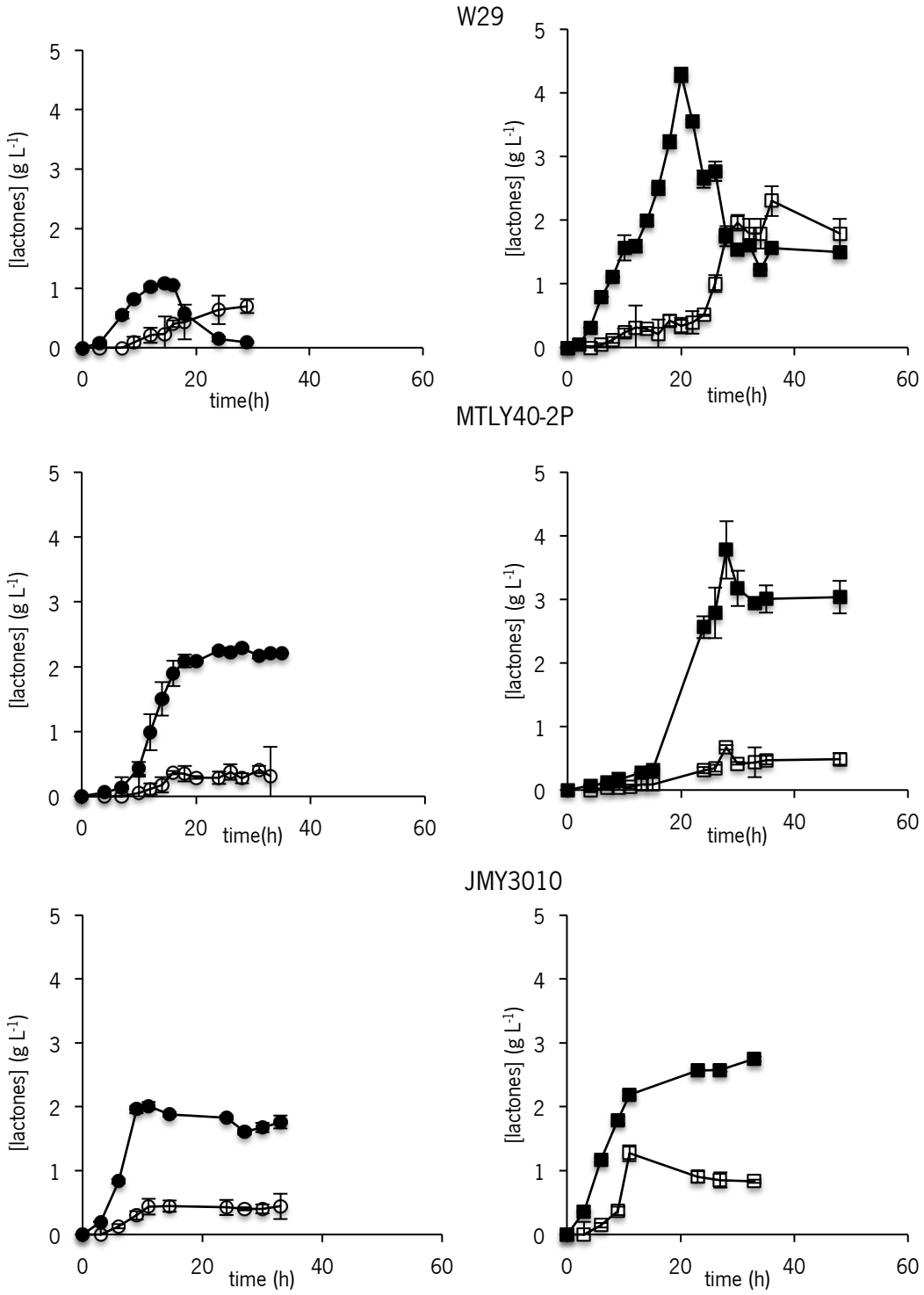


Figure 6.1 - Effect of substrate concentration in γ-decalactone (filled symbols) and 3-hydroxy-γ-decalactone (open symbols) production: (●) 30 g L^{-1} of castor oil; (■) 60 g L^{-1} of castor oil. Data are presented as mean and standard deviation of two independent experiments.

The kinetic profile of γ-decalactone production for the WT strain showed a maximum and after

a decrease in the aroma concentration, that is typical for this strain in batch cultures (Gomes et al., 2010). On the contrary, for mutant strains there was practically no significant decrease of the aroma at the end of the experiment, except for the MTYL40-2P strain with 60 g L^{-1} castor oil where a 30% decrease in γ-decalactone concentration occurred.

As expected the MTYL40-2P strain exhibited higher γ-decalactone production than the W29 strain. Indeed disruption of the *POX3* gene coding the short-chain specific enzyme (Aox3) avoided degradation of the aroma or its precursor via continuation of β-oxidation after C_{10} level or lactone reconsumption. Once strains lack the short-chain Aox (Aox3), they consumed more slowly γ-decalactone (Waché et al. 2000b, 2001, 2002). However, while no reconsumption was observed at castor oil concentration of 30 g L^{-1}, slight reconsumption could be observed at 60 g L^{-1} of castor oil, probably due to the action of the residual Axo1 and Aox6. Nevertheless, the lack of γ-decalactone reconsumption in JMY3010 strain was unexpected as this strain was not modified for its β-oxidation and possibly one of the hydrolysis products of castor oil, rapidly generated by overexpressed *Lip2* gene, could be involved in the regulation of the β-oxidation pathway or other flux responsible for lactone reconsumption.

Since β-oxidation pathway may lead to the accumulation of other compounds derived from 4-hydroxydecanoic acid (precursor of γ-decalactone), the production of 3-hydroxy-γ-decalactone, was also analyzed (Fig. 6.1, open symbols). As expected higher hydroxy-lactone concentration was achieved for strains that were not manipulated at β-oxidation level (W29 and JMY3010). Moreover, for these two strains 3-hydroxy-γ-decalactone production increases as castor oil concentration increases from 30 g L^{-1} to 60 g L^{-1}. In this case, when more substrate is present the production of hydroxy-related lactones was increased due to the substrate availability, since the pathway is not blocked after C_{10} level. Also, for W29 strain 3-hydroxy-γ-decalactone increased as γ-decalactone disappeared. In mutants with *POX3* disrupted genes the production of hydroxylactone was minimized (MTLY40-2P). Waché et al. (2001) also reported a decrease in the production of hydroxylactones with *Δpox2Δpox3* mutants. 3-Hydroxy-γ-decalactone does not seem to be degraded in the same manner as γ-decalactone, since remained in the medium during all experiment.

Besides the differences observed in maximal aroma concentration obtained with the increase of castor oil concentration, the production rate was also changed (Fig. 6.2). For W29 strain, the increase in castor oil concentration led to an increase in aroma production rate (a 4-fold increase in maximum values), however there was a delay in the beginning of aroma production at 60 g L^{-1} castor oil compared to 30 g L^{-1}. Also an about 2-fold increase in the γ-decalactone consumption rate was

observed with the castor oil increase. For MTLY40-2P strain a lower initial (first 7 h) production rate was observed compared to W29 strain and this delay was more significant at high castor oil concentration. However, at 30 castor oil g L^{-1} higher values of production rate were reached than for W29 (c.a 2.5-fold). The increase of castor oil concentration for MTLY40-2P strain did not affect the maximum production rate attained but γ-decalactone consumption was observed (negative production rate).

As JMY3010 strain have an additional copy of *LIP2* gene, thus producing more lipase than the W29 and MTYL40-2P strains, a fast initial aroma production occurred particularly for 30 g L^{-1} castor oil. For W29 and JMY3010 stains, the increase of castor oil concentration leaded to an increase in the maximum production rate attained.

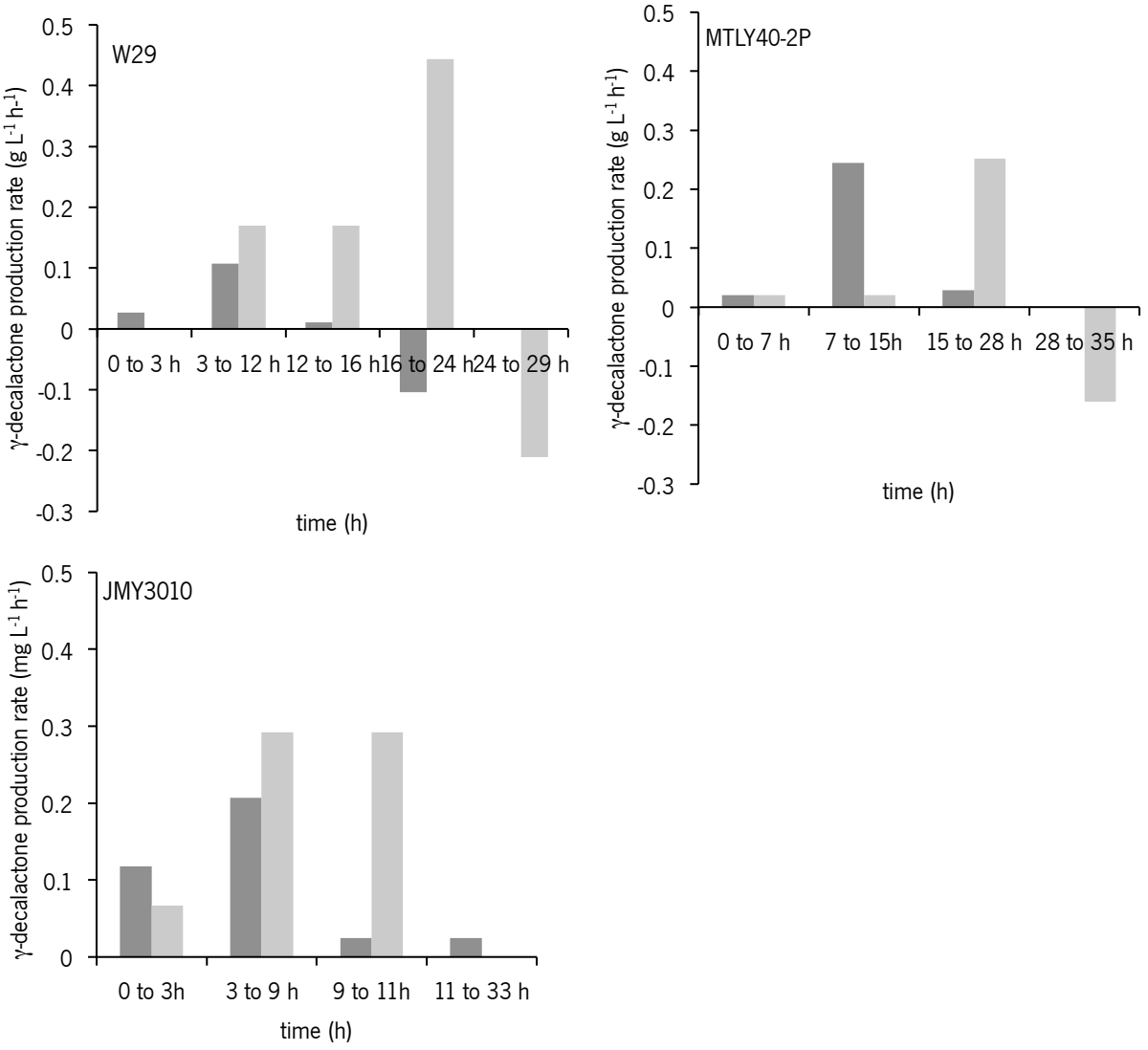


Figure 6.2 – γ-Decalactone production rate with different substrate concentration: (■) 30 g L^{-1} and (■) 60 g L^{-1}.

The lipase production was analyzed and higher lipase production was obtained with JMY3010 strain (2000 U L^{-1} to 3500 U L^{-1}) as expected. For W29 and MTLY40-2P strains a lower lipase production was observed (1000 U $L^{-1}$and 840 U L^{-1}, respectively).

These differences in extracellular lipase explain the delay observed in the γ-decalactone production observed for MTLY40-2P that was eliminated in JMY3010, overexpressing *LIP2* gene. The existence of a lag phase particularly in MTLY40-2P strain in γ-decalactone production can be attributed to the time necessary for the synthesis of lipases involved in the hydrolysis of castor oil, in order to enable the availability of ricinoleic acid (which is the substrate for the aroma synthesis) to the cells. This fact was also previously reported in Chapter 4 once the addition of an extracellular enzyme in the biotransformation medium resulted in a reduction of the lag phase observed for γ-decalactone production, since in these conditions the oil is earlier hydrolyzed into ricinoleic acid. In an industrial point of view, this process is not the most adequate since it is cost- and time-consuming. However, the use of an engineered strain overexpressing *LIP2* enzyme would bridge the gap of this problem by improving γ-decalactone production with no extra costs.

From Table 6.1 is clear that the highest aroma productivity was obtained for WT strain, since the highest aroma concentrations were achieved with this strain and MTLY40-2P, but due to the lag phase of this last, more time was needed to achieve the maximum concentration, leading to a lower global productivity.

For JMY3010 strain a higher production rate was observed but the maximal aroma concentration obtained was lower, leading to lower productivities.

Table 6.1 – Maximum γ-decalactone and 3-hydroxy-γ-decalactone productivity in batch cultures. Data are presented as the mean and standard deviation of two independent experiments

Strain	[CO] (g L^{-1})	γ-decalactone $Productivity_{maximum}$ (g $L^{-1}h^{-1}$)	3−hydroxy−γ-decalactone $Productivity_{maximum}$ (g $L^{-1}h^{-1}$)
W29	30	0.08 ± 0.01	0.02 ± 0.01
	60	0.21 ± 0.03	0.07 ± 0.01
MTLY40-2P	30	0.08 ± 0.01	0.01 ± 0.001
	60	0.14 ± 0.02	0.02 ± 0.001
JMY3010	30	0.13 ± 0.02	0.03 ± 0.001
	60	0.08 ± 0.02	0.12 ± 0.02

Concerning 3-hydroxy-γ-decalactone, the highest productivity was obtained with JMY3010 strain at higher CO concentration, contrarily to γ-decalactone, which showed a lower productivity under those conditions.

6.1.3.2 STEP-WISE FED-BATCH CULTURE

Yarrowia lipolytica can use γ-decalactone as a carbon source resulting in its complete disappearance after some hours of batch culture, as previously observed in the experiments with lower oil concentration for WT strain (Fig. 6.1). However, although this is avoided with an increase in the substrate concentration and with mutant strains, in the expectation to achieve higher γ-decalactone productivities, a step-wise fed-batch strategy was attempted in which 60 g L^{-1} of castor oil was added in two pulses to the bioreactor (Fig. 6.3).

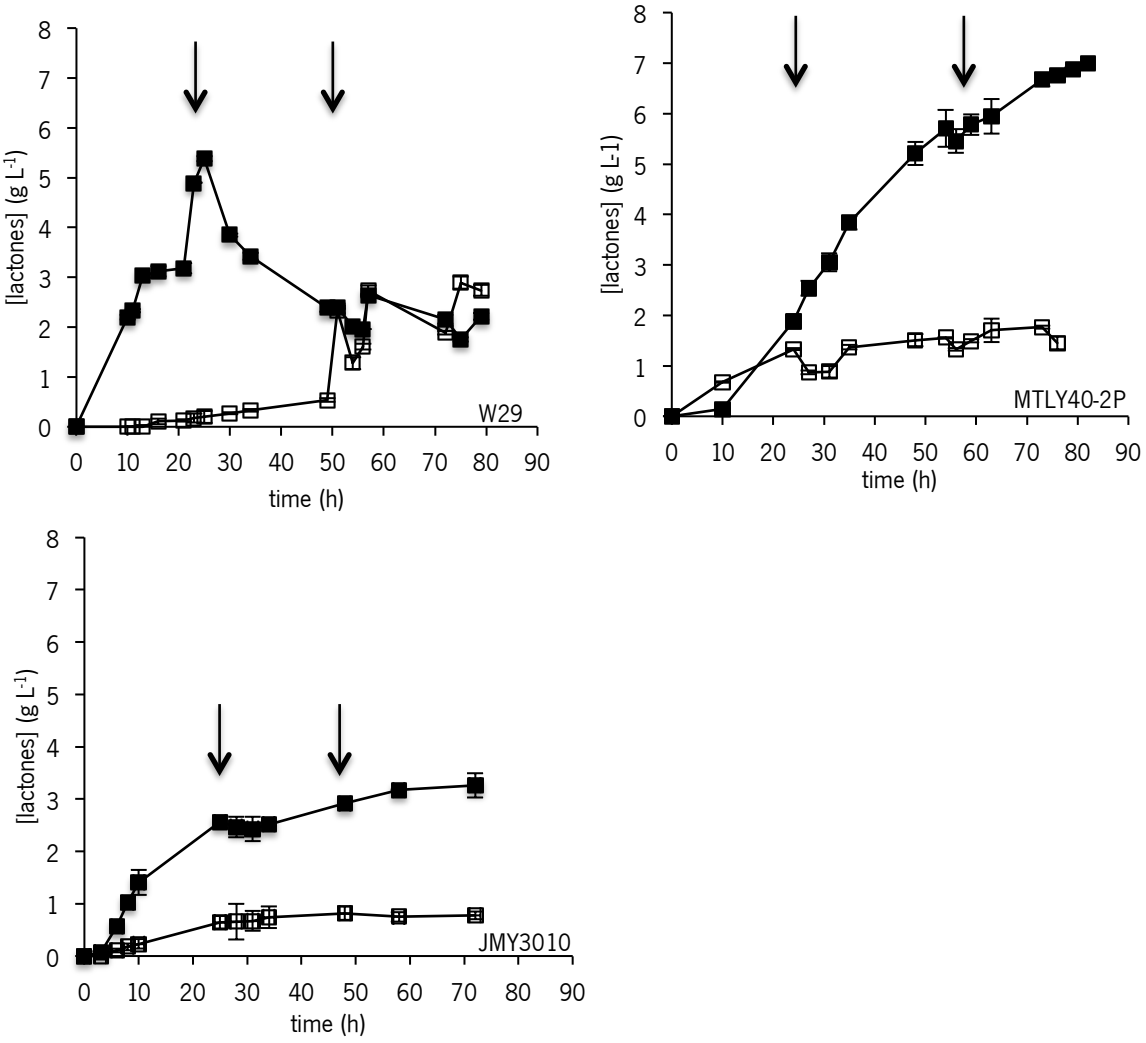


Figure 6.3 - γ-Decalactone (■) and 3-hydroxy-γ-decalactone (□) production in a step-wise fed-batch, after a batch phase with 60 g L^{-1} castor oil. The solid arrows indicate 60 g L^{-1} castor oil addition to the medium. Data are presented as mean and standard deviation of two independent experiments.

For W29 and JMY3010 strains no greater improvement in γ-decalactone concentration was achieved using step-wise fed-batch culture. On the other hand, a greater increase in aroma concentration was obtained for MTLY40-2P strain. With first substrate addition no decrease in aroma production was observed and the second substrate feed increased aroma production. In fact a 2-fold increase in γ-decalactone production was achieved with this strategy, leading to a maximum aroma concentration of 7 g L^{-1}.

Most of the papers reported an increase on γ-decalactone production in fed-batch when compared to batch (Lee et al., 1995). Lee et al. (1995) attempted a fed-batch strategy to produce γ-decalactone by *Sporobolomyces odorus* from castor oil hydrolysate as source of ricinoleic acid. High aroma production was also described for a three-step feeding of castor oil, in which a maximum concentration of γ-decalactone, 208 mg L^{-1}, was obtained over 168 h. Ambid et al. (2003) studied the γ-decalactone production with the same yeast, with the goal of increasing the process productivity, methyl ricinoleate additions with 24 h gaps were tested, and a considerably improvement in aroma production was found (142.7 mg L^{-1} instead of 47.7 mg L^{-1}) in a fed-batch operation.

Concerning the production of 3-hydroxy-γ-decalactone, it was observed that, in general, the concentrations obtained in fed-batch were similar with those obtained in batch for higher castor oil concentration (Fig. 6.1), except for MTLY40-2P strain since a higher hydroxylactone production was observed in fed-batch. In this case, the amount of castor oil added to the culture medium, after the second castor oil addition, was 3-fold the value in the batch culture. These results indicate that for this strain the level of the hydroxylactone produced may be castor oil concentration dependent above certain values.

From Tables 6.1 and 6.2 is possible to observe that in general, the productivity values of γ-decalactone obtained with step-wise fed-batch and batch where quite similar, slightly lower in the fed-batch cultures.

Table 6.2 – Maximum γ-decalactone and 3-hydroxy-γ-decalactone productivity in step-wise fed-batch cultures. Data are presented as the mean and standard deviation of two independent experiments

Strain	γ-decalactone productivity step-wise fed-batch (g $L^{-1}h^{-1}$)	3−hydroxy−γ-decalactone productivity step-wise fed-batch (g $L^{-1}h^{-1}$)
W29	0.22 ± 0.03	0.05 ± 0.01
MTYL40-2P	0.10 ± 0.02	0.03 ± 0.005
JMY3010	0.05 ± 0.01	0.02 ± 0.007

The highest γ-decalactone concentration was achieved with MTLY40-2P strain and around 1.7–fold improvement of maximum aroma concentration was obtained in step-wise fed-batch mode compared to batch culture (Table 6.1).

Regarding 3-hydroxy-γ-decalactone productivity, higher values were obtained in batch mode since more time was needed to achieve higher hydroxyl-lactone concentration in fed-batch, leading to a lower global productivity.

During biotransformations castor oil concentrations in the medium was measured by GC through fatty acids methyl esters quantification. Through batch phase substrate was completely consumed, remaining in the medium a concentration below 10 g L^{-1}, in all experiments. Figure 6.4 represent the substrate profile for step-wise fed-batch experiments with three strains.

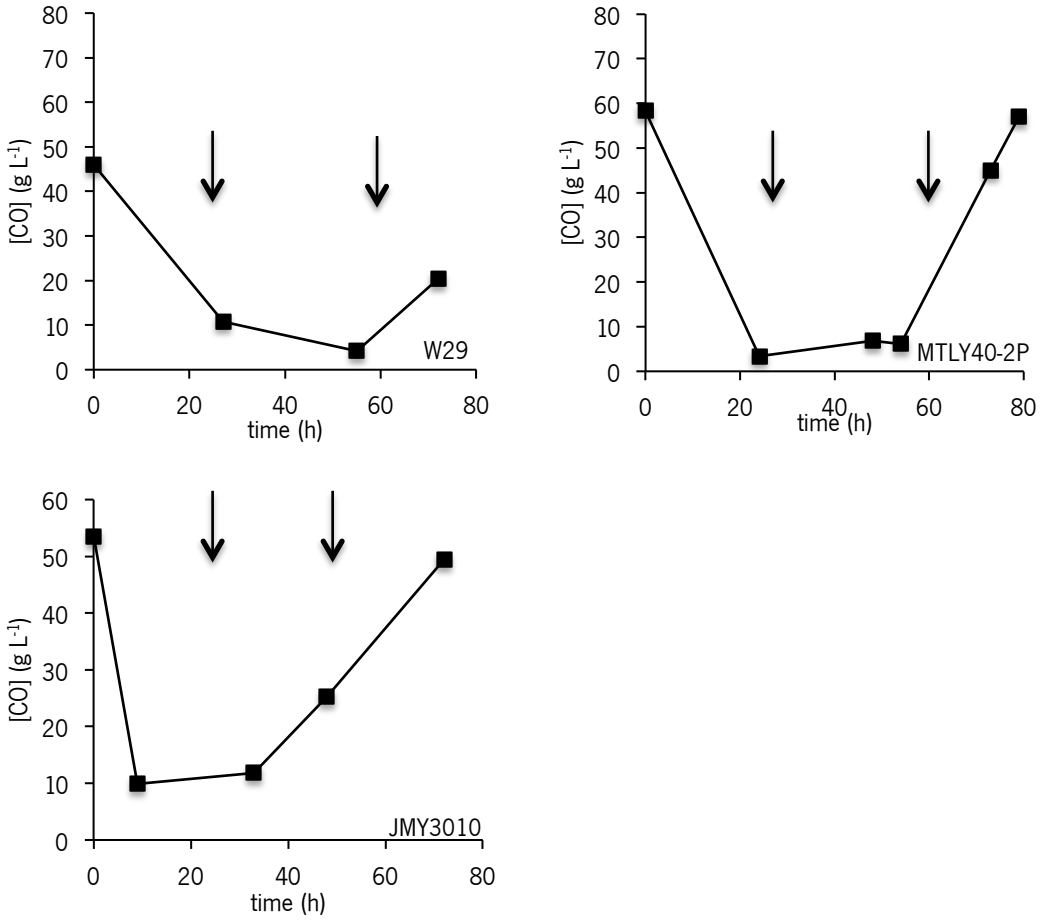


Figure 6.4 - Substrate profile for step-wise fed-batch experiments. The solid arrows indicate 60 g L^{-1} castor oil addition to the medium.

For W29 strain substrate concentration remains between 20 g L^{-1} and 5 g L^{-1} during all experiment, even after the two-substrate feeds. Similar results were observed for MTLY40-2P and JMY3010 strains, however after the second substrate addition a higher substrate concentration, around 60 g L^{-1}, accumulated in the medium.

In the step-wise fed-batch, the total yields for lactones production ranges from 0.03 g g^{-1} to 0.07 g g^{-1}. Regarding solely the conversion of the substrate to γ-decalactone, the yields range between 0.06 g g^{-1} and 0.01 g g^{-1}. These results are in accordance with the values found in the literature that normally range from 0.07 g g^{-1} to 0.012 g g^{-1}, depending on the strain, substrate type and concentration used (Waché et al., 2001, 2002; Le Do et al., 2013; Gomes et al., 2012).

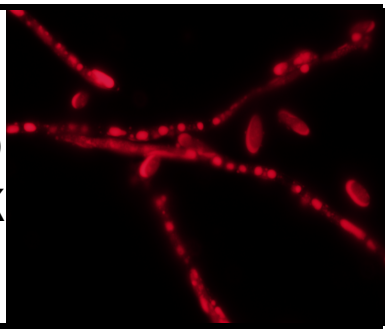
6.1.4 CONCLUSIONS

In this study, the effect of castor oil concentration in different strains genotypes was specified. γ-Decalactone production from castor oil by *Y. lipolytica* mutants has been analyzed and castor oil concentration of 60 g L^{-1} were shown to be the most adequate condition for γ-decalactone production in batch cultures. Our results confirm that for mutant strains with *POX2* and *POX3* disrupted genes there was practically no significant decrease of the aroma at the end of the experiment and the production of hydroxylactone was minimized.

The productivity values obtained with batch and step-wise fed-batch approach where quite similar, but a greater increase in aroma production was obtained with MTLY40-2P strain and around 1.7–fold improvement of maximum aroma concentration was obtained in step-wise fed-batch mode compared to batch culture.

Considering the impact of castor oil hydrolysis to improve γ-decalactone production, it would be very interesting to combine modification at *POX* level and *Lip2* overexpression.

FINAL CONCLUSIONS AND PERSPECTIVES FOR FUTURE WORK



The overall conclusions of the work described are herein presented as well as suggestions for future work related with this field of research.

7.1 FINAL CONCLUSIONS

γ-Decalactone production by biotransformation of castor oil using microorganisms is an attractive means to produce aroma compounds. Even more since these compounds are structurally identical with those present in natural sources and possess the "natural" label which is considered advantageous by the consumer preferences. Although, the scientific community has dedicated time and efforts around the production of "natural" aromas, the overall productivity is still very low.

The studies reported in this dissertation were primarily focused on the selection of different strategies to increase γ-decalactone production. Several approaches were used to optimize the aroma production but still, the process was quite slow and with reduced productivities. An insufficient oil hydrolysis was hypothesized and thus the use of exogenous lipase was tested. In CHAPTER 4 the results have shown that the use of Lipozyme TL IM® revealed to be an efficient way to hydrolyze 95.4 % of the castor oil. The impact of using castor oil previously hydrolyzed or adding the exogenous lipase in the beginning of the biotrasnformation was analyzed and compared with the results obtained in the biotransformation without exogenous lipase. As expected, the γ-decalactone production was faster when lipase was added into the medium but lower concentrations were obtained. Althought similar productivities were obtained with and without lipase addition. With these results in consideration it was concluded that the use of commercial lipase is not justified, in experiments with *Y. lipolytica* W29 free cells. One of the limitations in the development of an industrial process for γ-decalactone production is the toxicity towards the substrate and the lactone itself. The immobilization by adsorption of *Y. lipolytica* W29 in methyl polymetacrilate and DupUm® was studied and compared with free suspended cells. At first, both supports showed to be a good alternative for cell immobilization once the yeast cells covered the supports surface in a thick layer, allowing a good immobilization rate. When comparing the γ-decalactone production with immobilized cells and cells in suspension, it was concluded that the best approach was to adsorb *Y. lipolytica* cells on DupUM® and add the extracellular lipase Lipozyme TL IM® to the biotransformation medium, since a higher amount of γ-decalactone was obtained and the aroma remained in the medium, e.g., reconsumption was prevented. Furthermore, immobilized cells hold a stable γ-decalactone production after being stored for 30 d at 4 °C. Also, it is possible to reuse cells until three consecutive cycles of cultivation without comprising the γ-decalactone production, since the accumulation of the compound decreased only to 80 % of the value obtained in the first cycle. This

is a very promising result for γ-decalactone production, with potential to be used at an industrial level since the use of immobilized cells system will facilitate the conversion of a batch process into a continuous mode keeping high cell density and will allow easier recovery of metabolic products.

In CHAPTER 5, γ–decalactone production from castor oil by *Y. lipolytica* W29 free cells was studied in STR and airlift bioreactors, taking into account the effect of oxygen transfer rate and bioreactor design. An empirical correlation to predict k_La values as a function of operating conditions for both bioreactors was established. The correlation for k_La prediction, herein proposed, could be very useful for further work on the development of strategies for the optimization and scale-up of the processes where oxygen transfer is a limiting factor. Also, the direct influence of oxygen transfer rate on γ-decalactone and 3-hydroxy-γ-decalactone production was demonstrated. The results revelead that the maximum γ-decalactone concentration was achieved in airlift for lower k_La value, however, similar productivities values were obtained in both bioreactors. Thus, the use of airlift bioreactors, may offer an alternative system for aroma production at large scale, since do not require a mechanical action, which was proven in this work that may induce shear stress to the cells.

In CHAPTER 6, the effect of castor oil concentration in different *Y. lipolytica* strains genotypes was studied in detail. γ-Decalactone production from castor oil by *Y. lipolytica* mutant strains have been analyzed and castor oil concentration of 60 g L^{-1} is shown to be the most adequate condition for γ-decalactone production in batch cultures. Our results confirm that in the end of each experiment there is no significant decrease of the aroma for the mutant strain with genetic modifications in the β-oxidation pathway. Moreover, it was observed that the concentration of hydroxylactone was minimized in the mutant with the gene *POX3* disrupted. The productivity values obtained with batch and step-wise fed-batch approach where quite similar, but a 1.7–fold increase in aroma concentration was obtained when MTLY40-2P mutant was used in a step-wise fed-batch mode. The step-wise fed-batch culture improved γ-decalactone production for MTLY40-2P strain, with a final γ-decalactone concentration of 7 g L^{-1}.

In the context of this dissertation further research should be performed.

7.2 PERSPECTIVES FOR FUTURE WORK

Although the present work brings new insights on the biotechnological production of γ-decalactone contributing with some strategies to increase the production of aroma, there is still same room for further studies and developments. Considering the utility of hydrolyzing the castor oil and genetic modifications in the β-oxidation pathway (as MTLY40-2P strain) to improve the production, it would be very interesting to overexpress the native lipase of the cells or to express recombinant lipases with higher activity.

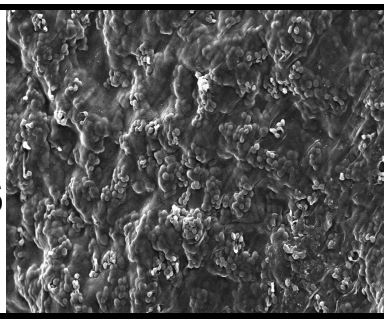
Another interesting assignment can pass throught the use of different supports and conditions regarding the immobilization of the MTLY40-2P strain, which was the strain with best production of aroma, or other derivate mutant, that produces higher amounts of γ-decalactone. In this context, the selection of other supports for immobilization by adsorption (such as polypropylene, polyurethane foam, among others) and techniques by inclusion (e.g. alginate, chitosan, agarose and k-carrageenan) may be performed. Flocculation can also be considered as a very promising technique for immobilization *Y. lipolytica* cells, by cloning genes known to promote flocculation in *S. cerevisiae*, a natural flocculant yeast.

An interesting culture strategy to investigate in this bioprocess is the continuous mode with immobilized cells, since it will decrease the toxicity of cells towards the substrate and the produced aroma. This will be particularly interesting to apply in pneumatically agitated bioreactors such as airlift bioreactor.

It is also of interest to construct a genome-scale model of *Y. lipolytica* that accommodates the γ-decalactone production pathway. This will lead to the formulation of rational strategies that envisage the production of γ-decalactone and the prediction of metabolic bottlenecks that will favour the accumulation of the desired compound.

Finally, in order to increase the viability of the process, it would be important to exploit the by-products accumulate during the lactone production, such as lipases, proteases and other lactones that are produced in considerable amounts. In addition, 3-hydroxy-γ-decalactone could be used to produce, by dehydration, two decenolides with aroma properties: dec-2-enolide (mushroom notes) and dec-3-enolide (fruity aroma).

REFERENCES



The following chapter lists all the references that contributed to the elaboration of this written work.

Aguedo, M. (2002a) Biotransformation de lipides en arômes par *Yarrowia lipolytica*: interactions avec le substrat, excrétion et toxicité des métabolites produits. PhD Thesis, Université de Bourgogne, Dijon, France.

Aguedo, M.; Beney, L.; Waché, Y.; Belin, J.-M.; Gervais, P. (2002b) Interaction of odorous lactones with phospholipids: implications in toxicity towards producing yeast cells. Biotechnol. Lett. 24:1975-1979.

Aguedo, M.; Beney, L.; Waché, Y.; Belin, J-M. (2003) Mechanisms underlying the toxicity of lactone aroma compounds towards the producing yeast cells. J. Appl. Microbiol. 94:258–265.

Aguedo, M.; Ly, M.H.; Belo, I.; Teixeira, J.A.; Belin, J.-M. ; Waché, Y. (2004) The use of enzymes and microorganisms for the production of aroma compounds from lipids. Food Technol. Biotechnol. 42:327- 336.

Aguedo, M.; Gomes, N.; García, E.E.; Waché, Y.; Mota, M.; Teixeira, J.A.; Belo, I. (2005a) Decalactone production by *Yarrowia lipolytica* under increased O_2 transfer rates. Biotechnol. Lett. 27:1617-1621.

Aguedo, M.; Waché, Y.; Belin, J.-M. Teixeira, J.A. (2005b) Surface properties of *Yarrowia lipolytica* and their relevance to γ–decalactone formation from methyl ricinoleate. Biotechnol. Lett. 27:417-422.

Ahlers, P.; Garofano, A.; Kerscher, S.; Brandt, U. (2000) Application of the obligate aerobic yeast *Yarrowia lipolytica* as a eukaryotic model to analyze Leigh Syndrome mutations in the complex I core subunits PSST and TYKY. Biochim. Biophys. Acta. 1459:258–265

Alchihab, M.; Destain, J.; Aguedo, M.; Majad, L.; Ghalfi, H.; Wathelet., J.-P.; Thonart, P. (2009) Production of γ-decalactone by a Psychrophilic and a Mesophilic Strain of the Yeast *Rhodotorula aurantiaca*. Appl. Biochem. Biotechnol. 158:41-50.

Alchihab, M.; Destain, J.; Aguedo, M.; Wathelet, J.P.; Thonart, P. (2010) The utilization of

gum tragacanth to improve the growth of *Rhodotorula aurantiaca* and the production of γ-decalactone in large scale. Appl. Biochem. Biotechnol. 162:233–241.

Amaral, A.L.; Ferreira, E.C. (2005) Activated sludge monitoring of a wastewater treatment plant using image analysis and partial least squares regression. Anal. Chim. Acta. 544:246–253.

Amaral, P.F.F.; da Silva, J.M.; Lehicky, M.; Barros-Timmons, A.M.V.; Coelho, M.A.Z.; Marrucho, I.M.; Coutinho, J.A.P.; (2006a) Production and characterization of a bioemulsifier from *Yarrowia lipolytica*. Proc. Biochem. 41:1894-1898.

Amaral, P.F.F.; Freire, M.G.; Rocha-Leão, M.H.M.; Marrucho, I.M.; Coutinho, J.A.P.; Coelho, M.A.Z. (2008) Optimization of oxygen mass transfer in a multiphase bioreactor with perfluorodecalin as a second liquid phase. Biotechnol. Bioeng. 99(3):588-598.

Ambid, C.; Carle, S.; De Billerbeck, G. (1999) Method for producing and extracting aromatic compounds. Patent WO 9954432.

Ambid, C.; Carle, S.; De Billerbeck, G. (2003) Process for producing and extracting aromatic compounds. US Patent 6518050B1.

Angerer, H.; Nasiri, H.R.; Niedergesäß, V.; Kerscher, S.; Schwalbe, H.; Brandt, U. (2012) Tracing the tail of ubiquinone in mitochondrial complex I. Biochim. Biophys. Acta. 1817: 1776–1784.

An, J.; Joo, Y-C.; Oh, D-K (2013) New biotransformation process for the production of the fragrance γ-dodecalactone from 10-hydroxystearate by permeabilized *Waltomyces lipofer* cells. Appl. Environ. Microbiol. 79:2636–2641.

Arctander, S. (1969) Perfume and flavour chemicals, vol. 1 (published by the author), Montclair, N. J.

Bakhuis, E.; Bos, P. (1969) Correlation between growth of *Candida lipolytica* and size of droplets in the hydrocarbon containing medium. Antonie van Leeuwenhoek 35: F47-F48.

Bar, R. (1989) Cyclodextrin-aided bioconversions and fermentations. Trends Biotechnol. 7:2–4

Barboza, M.; Zaiat, M.; Hokka, C.O. (2000) General relationship for volumetric oxygen transfer coefficient ($k_L a$) prediction in tower bioreactors utilizing immobilized cells. Bioprocess. Eng. 22:181-184.

Barth, G. (2013) *Yarrowia lipolytica*: Genetics, Genomics, and Physiology. Microbiology Monographs. Springer London, Limited.

Barth, G.; Beckerich, J.M.; Dominguez, A.; Stefan, K.; Ogrydziak, D.; Titorenko, V.; Gaillardin, C. (2003) Functional genetics of *Yarrowia lipolytica*. *In* Functional Genetics of Industrial Yeasts, de Winde JH (ed). Springer-Verlag: Berlin, Heidelberg, New York; 227–271.

Barth, G.; Gaillardin, C. (1997) Physiology and genetics of the dimorphic fungus *Yarrowia lipolytica.* FEMS Microbiol. Rev. 19:219-237.

Batzias, F.A.; Siontorou, C.G. (2012). Creating a specific domain ontology for supporting R&D in the science-based sector – The case of biosensors. Expert Systems with Applications 39, 9994–10015.

Beckerich, J. M.; Boisramé-Baudevin, A.; Gaillardin, C. (1998) *Yarrowia lipolytica*: a model organism for protein secretion studies. Internatl. Microbiol. 1: 123-130.

Bello, R.C.W.; Moo-Young, M. (1985) Prediction of the Volumetric Mass Transfer Coefficient in Pneumatic Contactors. Chem. Eng. Sci. 40:53-8.

Belo, I.; Pinheiro, R.; Mota, M. (1996) Pressurized biorreactors: Fundamentals and application. Dans: Comunicaciones del III Congresso Ibérico de Biotecnologia – BIOTEC'96. Fernández-Polanco, F., Benito, G.G., Encina, P.A.G. and Miranda, M.M.P. (Ed). Universidad de Valladolid, Valladolid, 35-37.

Belo, I.; Pinheiro, R.; Mota, M. (2003) Fed-batch cultivation of *Saccharomyces cerevisiae* in a

hyperbaric bioreactor. Biotechnol. Prog. 19: 665-671.

Benjamin, S.; Pandey, A. (1995) Optimization of a liquid media for lipase production by *Candida rugosa*. Bioresour. Technol. 55:167–170.

Beopoulos, A.; Mrozova, Z.; Thevenieau, F.; Le Dall, M-T.; Hapala, I.; Papanikolaou, S.; Chardot, T.; Nicaud, J-M. (2008) Control of lipid accumulation in the yeast *Yarrowia lipolytica*. App. Environ. Microbiol. 74:7779-7789.

Biryukova, E.N.; Medentsev, A.G.; Arinbasarova, A.Y.; Akimenko, V.K. (2006) Tolerance of the yeast *Yarrowia lipolytica* to oxidative stress. Microbiol. 75(3): 243-247.

Blažej, M.; Annus, J.; Markoš, J. (2004) Comparison of gassing-out and pressure-step dynamic methods for k_La measurement in an airlift reactor with internal loop. Chem. Eng. Res. Des. 82:1375-1382.

Blin-Perrin, C.; Molle, D.; Dufossé, L.; Le-Quere, J.-L. ; Viel, C. ; Mauvais, G. ; Feron, G. (2000) Metabolism of ricinoleic acid into γ-decalactone: β-oxidation and long chain acyl intermediates of ricinoleic acid in the genus *Sporidiobolus sp*. FEMS Microbiol. Lett. 188:69-74.

Brandt, U.; Abdrakhmanova, A.; Zickermann, V.; Galkin, A.; Dröse, S.; Zwicker, K.; Kerscher, S. (2005) Structure–function relationships in mitochondrial complex I of the strictly aerobic yeast *Yarrowia lipolytica*. Biochem. Soc. Trans.33: 840–844.

Brányik, T.; Vicente, A.A.; Oliveira, R.;Teixeira, J. A. (2004). Physicochemical surface properties of brewing yeast influencing their immobilization onto spent grains in a continuous reactor. Biotechnol. Bioeng. 88:84–93.

Bryers, J.; Characklis, W. (1981) Early fouling biofilm formation in a turbulent flow system: Overall kinetics. Wat. Res. 15:191-483.

Buckland, B.C.; Gbewonyo, K.; DiMasi, D.; Hunt, G.; Westerlield, G.; Nienow, A.W. (1988)

Improved performance in viscous mycelial fermentations by agitator retrofitting. Biotechnol. Bioeng. 31:737-42.

Capizzi, S.; Schwartzbrod, J. (2001). Surface properties of *Ascaris suum* eggs: hydrophobic potential and Lewis acid-base interactions. Colloids Surf., B. 22:99–105.

Carvalho, E.; Camarasa, E.; Meleiro, L.A.C.; Maciel Filho, R.; Domingues, A.; Vial, Ch.; Wild, G.; Poncin, S.; Midoux, N.; Bouillard, J. (2000) Development of a hydrodynamic model for air-lift reactors. Braz. J. Chem. Eng. 17:607-616.

Chang, J.; Fagarasanu, A.; Rachubinski, R.A. (2007) Peroxisomal peripheral membrane protein Yllnp1p is required for peroxisome inheritance and influences the dimorphic transition in the yeast *Yarrowia lipolytica*. Eukaryot. Cell 6: 1528–1537.

Cheetham, P. S. J.; Maume, K. A.; De Rooij, J. F. M. (1993) Method of producing γ-hydroxydecanoic acid or its lactone by feeding a ricinoleic acid source to *Sp. odorus* or *Rh. glutinis*. US Patent 5219742.

Cheremisinoff, N.P.; Gupta, R. (1983) Handbook of Fluids in Motion, Ann Arbor Science, Michigan.

Chisti, Y.; Moo-Young, M. (1989) On the calculation of shear rate and apparent viscosity in airlift and bubble column bioreactors. Biotechnol Bioeng 34:1391-2.

Chistia, Y.; Jauregui-Haza, U.J. (2002) Oxygen transfer and mixing in mechanically agitated airlift bioreactors. Biochem. Eng. J. 10:143–153.

Chisti, M.Y. (1989) Airlift bioreactors. Elsevier Science Publishers, Ltd., Barking, England.

Chow, C.K.; Palecek, S.P. (2004) Enzyme encapsulation in permeabilized *Saccharomyces cerevisiae* cells. Biotechnol. Prog. 20:449-456.

Christensen, M.W.; Kirk, O.; Pedersen, C. (2003) Process for immobilization of enzymes. USA Patent US20030203457.

Coelho, M.A.Z.; Belo, I.; Pinheiro, R.; Amaral, A.L.; Mota, M.; Coutinho, J.A.P.; Ferreira, E.C. (2004) Effect of hyperbaric stress on yeast morphology: study by automated image analysis Appl. Microbiol. Biotechnol.66: 318–324.

Coelho, M.A.Z.; Coutinho, J.A.P.; Ferreira, E.C.; Mota, M.; Belo, I. (2007) Analysis of the effects of hyperbaric gases on *S. cerevisiae* cell cycle through a morphological approach. Proc. Biochem. 42:1378–1383.

Corzo, G.; Revah, S. (1999) Production and characteristics of the lipase from *Yarrowia lipolytica* 681. Bioresour. Technol. 70:173-180.

Cruz, J.M.; Dominguez, J.M.; Dominguez, H. (2000) Dimorphic behavior of *Debaryomyces hansenii* grown on barley bran acid hydrolyzates. Biotechnol Lett 22: 605-610.

Czaczyk, K.; Myszka, K. (2007). Biosynthesis of extracellular polymeric substances (EPS) and its role in microbial biofilm formation. Pol. J. Environ. Stud. 16:799–806.

Domínguez, A.; Deive, F.J.; Sanromán, M.A.; Longo, M.A. (2003) Effect of lipids and surfactants on extracellular lipase production by *Yarrowia lipolytica*. J. Chem. Technol. Biotechnol. 78:1166–1170.

Doyle, R.J.; Rosenberg, M. (1990). Microbial cell surface hydrophobicity. American Society for Microbiology, Washington, D.C.

Dufossé, L. (1993) Production de lactones par des levures appartenant au genre *Sporodiobolus*. PhD Thesis, Université de Bourgogne, Dijon, France.

Dufossé, L.; Feron, G.; Mauvais, G.; Bonnarme, P.; Durand, A.; Spinnler, H.-E. (1998) Production of γ-decalactone and 4-hydroxy-decanoic acid in the genus *Sporodiobolus*. J. Ferment.

Bioeng. 86(2):169- 173.

Dufossé, L.; Souchon, I.; Feron, G.; Latrasse, A.; Spinnler, H.E. (1999) In situ detoxification of the fermentation medium during γ-decalactone production with the yeast *Sporidiobolus salmonicolor*. Biotechnol. Process. 15:135–139.

Dujon, B.; Sherman, D.; Fisher, G.; Durens, P.; Casaregola, S.; Lafontain, I.; de Montigny, J.; Marck, C.; Neuveglise, C.; Talla, E.; Goffard, N.; Frangeul, L.; Aigle, M.; Anthouard, V.; Babour, A.; Barbe, V.; Barnay, S.; Blanchin, S.; Beckerich, J.M.; Beyne, E.; Bleykasten, C.; Boisrame, A.; Boyer, J.; Cattolico, L.; Confanioleri, F.; de Daruvar, A.; Despons, L.; Fabre, E.; Fairhead, C.; Ferry-Dumazet, H.; Groppi, A.; Hantraye, F.; Hennequin, C.; Jauniaux, N.; Joyet, P.; Kachouri, R.; Kerrest, A.; Koszul, R.; Lemaire, M.; Lesur, I.; Ma, L.; Huller, H.; Nicaud, J.M.; Nikolski, M.; Oztas, S.; Ozier-Kalageropoulos, O.; Pellenz, S.; Potier, S.; Richard, G.F.; Straub, M.L.; Suleau, A.; Swennen, D.; Tekaia, F.; Wesolowski-Louvel, M.; Westhof, E.; Wirth, B.; Zeniou-Meyer, M.; Zivanovic, I.; Bolotin- Fukuhara, M.; Thierry, A.; Bouchier, C.; Caudron, B.; Scarpelli, C.; Gaillardin, C.; Waissenbach, J.; Wincker, P.; Souciet, J.L. (2004) Genome evolution in yeasts. Nature. 430:35-44.

Dumont, E.; Delmas, H. (2003) Mass transfer enhancement of gas absorption in oil-in-water systems: a review. Chem. Eng. Process. 42:419-438.

Endrizzi-Joran, A.; Awadé, A. C.; Belin, J.-M. (1993) Presumptive involvement of methyl ricinoleate β-oxidation in the production of γ-decalactone by the yeast *Pichia guilliermondii*. FEMS Microbiol. Lett. 114:153-160.

Endrizzi-Joran, A. (1994) PhD thesis, Université de Bourgogne, Dijon, France.

Endrizzi-Joran, A.; Belin, J.-M. (1995) Bioconversion of methyl ricinoleate to 4-hydroxy-decanoic acid and to γ-decalactone by yeasts of the genus *Candida*. J. Basic Microbiol. 35:285–292

Endrizzi-Joran, A.; Pagot, Y.; Le Clainchain, A.; Nicaud, J.M.; Belin, J.M. (1996) Production of lactones and peroxisomal β-oxidation in yeasts. Crit. Rev. Biotech. 16:301-329.

Farbood, M. I.; Willis, B. J. (1983) Production of γ-decalactone. Patent WO 8301072.

Farbood, M. I.; Willis, B. J. (1985) Optically active γ-hydroxy-decanoic acid preparation by cultivating microorganism to hydrolyse castor oil and subjecting to β-oxidation. US Patent 4560656.

Farbood, M.; Morris, J.A.; Sprecker, M.A.; Bienkowski, L.J.; Miller, K.P.; Vock, H.M.; Hagerdorn, M.L. (1989) Process for preparing compositions containing unsaturated lactones, products there by and organoleptic uses of said products. European patent 0 354 000.

Farbood, M. I.; Morris, J. A.; Sprecker, M. A.; Bienkowski, L. J.; Miller, K. P.; Vock, M. H.; Hagedorn, M. L. (1990) Process for preparing compositions containing unsaturated lactones and organoleptic uses of the lactones. European Patent 354000.

Fabritius, D.; Schafer, H-J.; Steinbchel, A. (1998) Bioconversion of sunflower oil, rapeseed oil and ricinoleic acid by *Candida tropicalis* M25. Appl. Microbiol. Biotechnol. 50:573–578

Fantin, G.; Fogagnolo, M.; Guerrini, A.; Medici, A.; Pedrini, P.; Fontana, S. (2001) Enantioselective hydrolyses with *Yarrowia lipolytica*: a versatile strain for esters, enol esters, epoxides, and lactones. Tetrahedron 12:2709–2713.

Fernandez-Lafuente, R. (2010) Lipase from *Thermomyces lanuginosus*: Uses and prospects as an industrial biocatalyst. J. Mol. Catal. B: Enzym. 62:197–212.

Feron, G.; Bonnarme, P.; Durant, A. (1996a) Prospects for the microbial production of food flavors. Trends Food Sci. Technol. 7:285-293.

Feron, G.; Dufossé, L.; Pierard, E.; Bonnarme, P.; Le Quere, J.-L.; Spinnler, H.-E. (1996b) Production, identification and toxicity of γ-decalactone and 4-hydroxydecanoic acid from *Sporidiobolus spp.*. Appl. Environ. Microbiol. 62(8):2826-2831.

Feron, G.; Dufossé, L.; Mauvais, G.; Bonnarme, P.; Spinnler, H.E. (1997) Fatty acid accumulation in the yeast *Sporidiobolus salmonicolor* during batch production of γ-decalactone. FEMS

Microbiol. Lett. 149:17–24.

Feron, G.; XiaoDong, W.;Viel, C.; Blin-Perrin, C.; Mauvais, G.; Cachon, R.; Diviés, C. (2002) Influence of a reducing environment on the bioconversion of fatty acids to γ-decalactone with *Sporidiobolus ruinenii*: Cellular and Enzymatic Approaches. *In* Flavour Research at the Dawn of the Twenty-First Century – 10th Weurman Flavour Research Symposium, J. L. Le Quere, P. X. Etievant (Eds.), Editions Tec & Doc, Paris.

Fickers, P.; Benetti, P.-H.; Waché, Y.; Marty, A.; Mauersberger, S.; Smit, M. S.; Nicaud, J.-M. (2005) Hydrophobic substrate utilization by the yeast *Yarrowia lipolytica*, and its potential applications. FEMS Yeast Res. 5:527-543.

Fickers, P.; Destain, J.; Thonard, P. (2009) Improvement of *Yarrowia lipolytica* lipase production by fed-batch fermentation. J. Basic Microbiol. 49:212-215.

Fishbein, W.N.; Bessman, S.P. (1966) Purification and properties of an enzyme in human blood and rat liver microsomes catalyzing the formation and hydrolysis of γ-lactones. I. Tissue location, stoichiometry, specificity, distinction from esterase. J. Biol. Chem. 241:4835-4841.

Fletcher, M. (1977) The effects of culture concentration and age, time, and temperature on bacterial attachment to polystyrene. Can. J. Microbiol. 23:1–6.

Foglia, T.A.; Zones, K.C.; Sonnet, P.E. (2000) Selectivity of lipases: isolation of fatty acids from castor, coriander, and meadowfoam oils. Eur. J. Lipid Sci. Technol. 102:612–61.

Freeman, A.; Lilly, M. D. (1998) Effect of processing parameters on the feasibility and operational stability of immobilized viable microbial cells. Enzyme Microb. Technol. 23:335-345.

Fuganti, C.; Servi, S.; Barbeni, M.; cabella, P. (1993). New avenues in natural products: strategies in the biogeneration of flavors. *In* Studies in natural products chemistry. AttarurRahman (Ed). Elsevier Science Publishers, Amsterdam, 295-345.

Gaillardin, C.; Neuveglise, C.; Kerscher, S.; Nicaud, J.M. (2012) Mitochondrial genomes of yeasts of the *Yarrowia* clade. FEMS Yeast Res. 12: 317–331.

García, E. E.; Belin, J.-M.; Waché, Y. (2007) Use of a Doehlert factorial design to investigate the effects of pH and aeration on the accumulation of lactones by *Yarrowia lipolytica*. J. Appl. Microbiol. 103:1508- 1515.

García, E. E. (2008) Aspects de la dégradation de substances hydrophobes en composés d'arômes par la levure *Yarrowia lipolytica*. PhD Thesis, Université de Bourgogne, Dijon, France.

García, E. E.; Aguedo, M.; Gomes, N.; Choquet, A.; Belo, I.; Teixeira, J. A.; Belin, J.-M.; Waché, Y. (2009) Production of 3-hydroxy-γ-decalactone, the precursor of two decenolides with flavouring properties, by the yeast *Yarrowia lipolytica*. J. Mol. Catal. B: Enzym. 57:22-26.

Garcia-Ochoa, F.; Gomez E. (2009) Bioreactor scale-up and oxygen transfer rate in microbial processes: An overview. Biotechnol. Adv .27: 153-176.

Gatfield, I.L. (1988) Production of flavor and aroma compounds by biotechnology. Food Technol. 190:110–123.

Gatfield, I. L.; Güntert, M.; Sommer, H.; Werkhoff, P. (1993) Some aspects of the microbiological production of flavour-active lactones with particular reference to γ-decalactone. Chem. Microbiol. Technol. Lebensm. 15:165-170.

Genisheva, Z.; Mussatto, S.; Oliveira, J.M.; Teixeira, J. A. (2011) Evaluating the potential of winemaking residues and corn cobs as support materials for cell immobilization for ethanol production. Ind. Crop. Prod. 34:979–985.

Glasbey, C.A.; Horgan, G.W. (1995) Image Analysis for the Biological Sciences. Wiley, Chichester.

Gocho, S.; Rumi, K.; Tsuyoshi, K. (1998) Process for the production of γ-decalactone. US

Patent 5763233 A.

Gonçalves, C.; Rodriguez-Jasso, R.M.; Gomes, N.; Teixeira, J.A.; Belo, I. (2010) Adaptation of dinitrosalicylic acid method to microtiter plates. Anal. Methods 2:2046-2048.

Gomes, N.; Aguedo, M.; Teixeira, J. A.; Belo, I. (2007) Oxygen mass transfer in a biphasic medium: Influence on the biotransformation of methyl ricinoleate into γ–decalactone by the yeast *Yarrowia lipolytica*. Biochem. Eng. J. 35:380-386.

Gomes, N.; Teixeira, J.A.; Belo, I. (2010) The use of methyl ricinoleate in lactone production by *Yarrowia lipolytica*: aspects of bioprocess operation that influence the overall performance. Biocatal. Biotransform. 28(4):227–234

Gomes, N. (2011a) Biotransformation of ricinoleic acid into γ-decalactone by Yarrowia lipolytica: Bioprocess optimization. PhD thesis, Braga, Portugal.

Gomes, N.; Gonçalves, C.; García-Román. M.; Teixeira, J.A.; Belo, I. (2011b) Optimization of a colorimetric assay for yeast lipase activity in complex systems. Anal. Methods 3:1008–1013.

Gomes, N.; Teixeira, J.; Belo, I. (2012) Fed-batch versus batch cultures of *Yarrowia lipolytica* for γ-decalactone production from methyl ricinoleate. Biotechnol. Lett. 34(4):649-54.

Gómez-Díaz, D.; Navaza, J. M.; Sanjurjo, B. (2008) Interfacial area evaluation in a bubble column in the presence of a surface-active substance: Comparison of methods. Chem. Eng. J. 144:379-385.

Gómez-Díaz, D.; Gomes, N.; Teixeira, J. A.; Belo, I. (2009) Oxygen mass transfer to emulsions in a bubble column reactor. Chem. Eng. J. 152: 354-360.

Gopinath, M.; Vijayakumar, L.; Dhanasekar, R.; Viruthagiri, T. (2008) Microbial biosynthesis of γ– decalactone and its applications – a review. Global J. Biotech. Biochem. 3(2):60-68.

Goswami, D.; Sen, R.; Basu, J.K.; De, S. (2009) Maximization of bioconversion of castor oil into ricinoleic acid by response surface methodology. Bioresour. Technol. 100:4067–4073.

Gryta, M. (2002). The assessment of microorganism growth in the membrane distillation system. Desalination, 142, 79–88.

Groboillot, A.; Boadi, D. K.; Proncelet, D.; Neufeld, R. J. (1994) Immobilization of cells for application in the food industry. Crit. Rev. Biotechnol. 14:75-107.

Groguenin, A.; Waché, Y.; García, E. E.; Aguedo, M.; Husson, F.; LeDall, M. T.; Nicaud, J.-M., Belin, J.-M. (2004) Genetic Engineering of the β-oxidation pathway in the yeast *Yarrowia lipolytica* to increase the production of aroma compounds. J. Mol. Catal. B: Enzymatic 28:75-79.

Gordillo, M.A.; Obradors, N.; Montesinos, J.L.; Valero, F.; Lafuente, J.; Sola, C. (1995) Stability studies and effect of the initial oleic acid concentration on lipase production by *Candida rugosa*. Appl. Microbiol. 43:38–41.

Gunkel, K.; van der Klei, I.J.; Barth, G.; Veenhuis, M. (1999) Selective peroxisome degradation in *Yarrowia lipolytica* after a shift of cells from acetate/oleate/ethylamine into glucose/ammonium sulfate-containing media. FEBS Lett. 451:1–4.

Guo, Y.; Feng, C.; Song, H.; Wang, Z.; Ren, Q.; Wang, R. (2011) Effect of *POX3* gene disruption using self-cloning CRF1 cassette in *Yarrowia lipolytica* on the γ-decalactone production. World J. Microb. Biot. doi:10.1007/s11274-011-0758-7

Guo, Y.; Song, H.; Wang, Z.; Ding, Y. (2012) Expression of *POX2* gene and disruption of *POX3* genes in the industrial *Yarrowia lipolytica* on the γ-decalactone production. Microbiol. Res. 167:246–252.

Haffner, T.; Tressl, R. (1996) Biosynthesis of (R)-γ-decanolactone in the yeast *Sporobolomyces odorus*. J. Agric. Food Chem. 44:1218-1223.

Hattori, T.; Furusaka. C. (1960) Chemical activities of *E. coli* adsorbed on a resin. Biochem. J. 48:831-837.

Hegedus, D.D.; Khachatourians, G.G. (1988) Production of an extracellular lipase by *Beauveria Bassiana*. Biotechnol. Lett. 9:637–642.

Hettema, E.H.; Tabak, H.F. (2000) Transport of fatty acids and metabolites across the peroxisomal membrane. Biochim. Biophys. Acta, 1486:18–27.

Holcberg, I.B.; Margalith, P. (1981) Alcoholic fermentation by immobilized yeast at high sugar concentration. Eur. J. Appl. Microbiol. Biotechnol. 13:133-137.

Holzschu, D.; Chandler, F.; Ajello, L.; Ahearn, D. (1979) Evaluation of industrial yeasts for pathogenicity. Sabouraud.17: 71–78.

Hsu, J. W.; Speers, R. A.; Paulson, A. T. (2001) Modeling of orthokinetic flocculation of *Saccharomyces cerevisiae*. Biophys. Chem. 94:47-58.

Iacazio, G.; Martini, D.; Faure, B.; N'Guyen, M. H. (2002) Isolation and characterization of 8-hydroxy-3Z,5Z- tetradecadienoic acid, a putative intermediate in *Pichia guilliermondii* γ-decalactone biosynthesis from ricinoleic acid. Microbiol. Lett. 209:57-62.

Kapfer, G.-F.; Berger, R.G.; Drawert, F. (1989) Production of 4-decanolide by semicontinuous fermentation of *Tyromyces sambuceus*. Biotechnol. Lett. 11:561-566.

Kautola, H.; Rymowicz, W.; Linko, Y.; Linko, P. (1991) Production of citric acid with immobilized *Yarrowia lipolytica*. Appl. Microbiol. Biotechnol. 35:447-449.

Kawasse, F. M.; Amaral P. F.; Rocha-Leão, M. H. M.; Amaral, A. L.; Ferreira, E. C.; Coelho, M. A. Z. (2003) Morphological analysis of *Yarrowia lipolytica* under stress conditions through image processing. Bioproc. Biosyst. Eng. 25:371-375.

Kawase, Y.; Moo-Young, M. (1987) Influence of antifoam agents on gas hold-up and mass transfer in bubble columns with non-newtonian fluids. Appl. Microbiol. Biotechnol. 27: 159-167.

Kerscher, S.; Grgic, L.; Garofano, A.; Brandt, U. (2004) Application of the yeast *Yarrowia lipolytica* as a model to analyse human pathogenic mutations in mitochondrial complex I (NADH:ubiquinone oxidoreductase). Biochim. Biophys. Acta. 1659: 197–205.

Kim, H. S.; Ju, J. Y.; Suh, J. H.; Shin, C. S. (1999) Optimized fed-batch fermentation of L-β-hydroxy isobutyric acid by *Yarrowia lipolytica*. Bioproc. Biosys. Eng. 20(3):189-193.

Kim, T.-H.; Oh, Y.-S.; Kim, S.-J. (2000) The possible involvement of the cell surface in aliphatic hydrocarbon utilization by an oil-degrading yeast. J. Microbiol. Biotechnol. 10:333-337.

Kim, B. S.; Hou, C. T. (2006) Production of lipase by high cell density fed-batch culture of *Candida cylindracea*. Bioproc. Biosys. Eng. 29:59-64.

Kinoshita, S.; Udaka, S.; Shimono, M. (1957) Studies on the amino acid fermentation. Part I. Production of L-glutamic acid by various microorganisms. J. Gen. Appl. Microbiol.3:193-20.

Kionka, C.; Kunau, W.-H. (1985) Inducible L-oxidation pathway in *Neurospora crassa*. J. Bacteriol. 161:153:157.

Kirk, O. (1979) Encyclopedia of Chemical Technology. John Wiley and Sons, New York.

Klein, J.; Ziehr, H. (1990) Immobilization of microbial cells by adsorption. J. Biotechnol. 16:1–15.

Kolot, F. B. (1981) Microbial carriers-strategy for selection. Process Biochem. 16:2-9.

Kondo, A.; Liu, Y.; Furuta, M.; Fujita, Y.; Matsumoto, T.; Fukuda, H. (2000) Preparation of high activity whole cell biocatalyst by permeabilization of recombinant flocculent yeast with alcohol. Enzyme Microb. Technol. 27:806–811.

Kosaric, N.; laszczyk, R. (1990) Wastewater treatment by immobilized cells. Boca Raton: CRC Press.

Koukoutas, Y.; Mcerlean, C.; Kanellaki, M.; Hack, C. J.; Marchant, R.; Banat, I. M.; Koutinas, A. A. (2004) High temperature wine making using the thermotolerant yeast strain *Kluyveromyces marxianus* IMB3. Appl. Biochem. Biotechnol. 112:25-35.

Kreger van Rij, N. (1984) The yeasts, a taxonomic study. Elsvier Science & Technology.

Krings, U.; Berger, R. G. (1998) Biotechnological production of flavours and fragrances. Appl. Microbiol. Biotechnol. 49:1-8.

Kulkarmi, N. (2002) Studies of Lipase Enzyme from *Pseudomonas Fluorescens* NS2W. Ph.D. Thesis, University of Pune, India.

Kulkarni, S.R.; Pandit, A.B. (2005) Enzymatic hydrolysis of castor oil: an approach for rate enhancement and enzyme economy. Indian J. Biotechnol. 4:241–245.

Kumin, B.; Munch, T. (1998) Microbiological process for producing γ-decalactone. US Patent 5849551.

Kun, H.;Yin, Y.; Hao, Z.; Ximin, Z. (2011) Optimization of hydrolysis of rapeseed oil with Lipozyme TL IM catalysis by response surface methodology. China Oils and Fats.

Kwok, D.Y.; Neumann, A.W. (1999). Advances in Colloid and Interface Science, 81:167–249.

Kyong, S.H.; Shin, C.S. (2000) Optimized production of L-β-hydroxybutyric acid by a mutant of *Yarrowia lipolytica*. Biotechnol. Lett. 22:1105-1110.

Lakshminara, G.; Subbarao, R.; Sastry, Y.S.R.; Kale, V.; Rao, T.C.; Gangadhar, A. (1984) High pressure splitting of castor oil. J. Am. Oil Chem. Soc. 61:1204–1206.

Latrasse, A.; Guichard, E.; Piffaut, C.; Fournier, N.; Dufoss, L. (1993) Chirality of the γ-lactones formed by *Fusarium poae* INRA 45. Chirality 5:379–384.

Le Do, T.T.; Vu, N.T.; Phan-Thi, H.; Cao-Hoang, L.; Ta, T.M.N.; Waché, Y.; Nguyen, T.H.T. (2013) Traditional fermented sausage 'Nem chua' as a source of yeast biocatalysts efficient for the production of the aroma compound γ-decalactone. Int. J. Food Sci. Technol. 49:1099–1105.

Lee, S.-L.; Chou, C.-C. (1994) Growth and production of γ-decalactone and cis-6-dodecen-4-olide by *Sporobolomyces odorus* in the presence of fatty acids and oils. J. Ferment. Bioeng. 78:114-116.

Lee, S.-L.; Lin, S.-J.; Chou, C.-C. (1995) Growth and production of γ-decalactone by *Sporobolomyces odorus* in jar fermentors as affected by pH, aeration and fed-batch technique. J. Ferment. Bioeng. 80:195- 199.

Lee, S. L.; Cheng, H. Y.; Chen, W. C.; Chou, C. C. (1998) Production of γ -decalactone from ricinoleic acid by immobilized cells of *Sporidiobolus salmonicolor*. Proc. Biochem. 33:453-459.

Lee, S. L.; Cheng, H. Y.; Chen, W. C.; Chou, C. C. (1999a) Effect of physical factors on the production of γ-decalactone by immobilized cells of *Sporidiobolus salmonicolor*. Proc. Biochem. 33:845-850.

Lee, Y.J.; Kim, C.S.; Oh, D.K. (2004) Lactulose production by β-galactosidase in permeabilized cells of *Kluyveromyces lactis*. Appl. Microbiol. Biotechnol. 64:787-793.

Léonard, A.; Dandoy, P.; Danloy, E.; Leroux, G.; Meunier, C.F.; Rookeza, J.C.; Su, B.-L. (2011) Whole-cell based hybrid materials for green energy production, environmental remediation and smart cell-therapy. Chem. Soc. Rev. 40:860–885.

Leung, R.; Poncelet, D.; Neufeld, R. J. (1997) Enhancement of Oxygen Transfer Rate Using Microencapsulated Silicone Oils as Oxygen Carriers. J. Chem. Technol. Biotechnol. 68: 37–46.

Lima, V.M.G.; Krieger, N.; Sarquis, M.I.M.; Mitchell, D.A.; Ramos, L.P. (2003) Effect of nitrogen and carbon sources on lipase production by *Penicillium aurantiogriseum*. Food Technol. Biotechnol. 41: 105-110.

Lin, S.-J.; Lee, S.-L.; Chou, C.-C. (1996) Effects of various fatty acid components of castor oil on the growth and production of γ-decalactone by *Sporobolomyces odorus.* J. Ferment. Bioeng. 82(1):42-45.

Liu, Y. (1995). Adhesion kinetics of nitrifying bacteria on various thermoplastic supports. Colloids Surf. B. 5: 213-219.

Loira, N.; Dulermo, T.; Nicaud, J.M.; Sherman, D.J. (2012) A genome-scale metabolic model of the lipid-accumulating yeast *Yarrowia lipolytica*. BMC Syst. Biol. 6: 35.

Longo, M. A.; Sanromán, M. A. (2006) Production of food aroma compounds: Microbial and enzymatic methodologies. Food Technol. Biotechnol. 335-353.

Luo, Y.S.; Wang, H.J.; Gopalan, K.V.; Srivastava, D.K.; Nicaud, J-M.; Chardot, T. (2000) Purification and characterization of the recombinant form of Acyl CoA oxidase 3 from the yeast *Yarrowia lipolytica*. Arch Biochem Biophys.384:1-8.

Luo, Y.S.; Nicaud, J.M.; Van Veldhoven, P.P.; Chardot, T. (2002) The acyl-CoA oxidases from the yeast *Yarrowia lipolytica*: characterization of Aox2p. Arch. Biochem. Biophys. 407:32-8.

Lowe, K.C. (2002) Perfluorochemical respiratory gas carriers: benefits to cell culture systems. J. Fluor. Chem. 118:19-26.

Madzak, C.; Gaillardin, C.; Beckerich, J.M. (2004). Heterologous protein expression and secretion in the non-conventional yeast *Yarrowia lipolytica*: a review. J. Biotechnol. 109:63-81.

Mafakher, L.; Mirbagheri, M.; Darvishi, F.; Nahvi, I.; Zarkesh-Esfahani, H.; Emtiazi, G. (2010) Isolation of lipase and citric acid producing yeasts from agro-industrial wastewater. N. Biotechnol. 27(4):337-340.

Mafu, A. K., Plumety, C., Deschênes, L. & Goulet, J. (2011) Adhesion of Pathogenic Bacteria to Food Contact Surfaces: Influence of pH of Culture. Int. J. Microb. doi:10.1155/2011/972494.

Maga, J. A. (1976) Lactones in foods. Crit. Rev. Food Sci. Nutri.8:1-56.

Malcata, F. X.; Hill, C. G. (1991) Use of a lipase immobilized in a membrane reactor to hydrolyze the glycerides of butter oil. Biotechnol. Bioeng. 38: 853-868.

Mansfeld, J.; Förster, M.; Hoffmann, T.; Schellenberger, A.; Dautzenberg, H. (1995) Coimmobilization of *Yarrowia lipolytica* cells and invertase in polyelectrolyte complex microcapsules. Enzyme. Microb. Tech. 17:11-17.

Marasco, E.; Schmidt-Dannert, C. (2003) Towards the biotechnological production of aroma and flavour compounds in engineered microorganisms. Appl. Biotechnol. Food Sci. Policy 1(3):145-157.

Margaritis, A.; Kilonzo, P.M. (2005) Production of ethanol using immobilized cell bioreactor system, In: Nedovic, V., Willaert, R. (Eds.) Applications of Cell Immobilization Biotechnology, Springer, Dordrecht.

Mather, J. P.; Roberts, P. E. (1998) Introduction to cell and tissue culture: Theory and technique, Plenum Press, New York.

Matos, M.; Alves, C.; Campos, J.L.; Brito, A.G.; Nogueira, R. (2011). Sequencing Batch Biofilm Reactor: from support design to reactor operation. Environ. Technol. 32 :1121–1129.

Matsumoto, T.; Takahashi, S.; Kaieda, M.; Ueda, M.; Tanaka, A.; Fukuda, H.; Kondo, A. (2001) Yeast whole-cell biocatalyst constructed by intracellular overproduction of *Rhizopus oryzae*

lipase is applicable to biodiesel fuel production. Appl. Microbiol. Biotechnol. 57:515-520.

Mesquita, D.P.; Amaral, A.L.; Ferreira, E.C.; Coelho, M.A.Z. (2009) Study of saline wastewater influence on activated sludge flocs through automated image analysis. J. Chem. Technol. Biotechnol. 84:554–560.

Mesquita, D.P.; Amaral, A.L.; Ferreira, E.C. (2011) Identifying different types of bulking in an activated sludge system through quantitative image analysis. Chemosphere 85:643–652.

Mesquita, D.P.; Leal. C.; Cunha, J.R.; Oehmen, A.; Amaral, A.L.; Reis, M.A.M.; Ferreira, E.C. (2013) Prediction of intracellular storage polymers using quantitative image analysis in enhanced biological phosphorus removal systems. Anal. Chim. Acta. 770:36–44.

Merchuk, J.C.; Ladwa, N.; Cameron, A.; Bulmer, M.; Pickett, A. (1994) Concentric-tube airlift reactors: Effects of geometrical design on performance. AI. Che. J. 40:1105-17.

Meyer, J. (1993) Gamma-decalactone microbial production from alkyl ricinoleate – by hydrolysis, β-oxidation, and chemical cyclisation of 4-hydroxy decanoic acid produced for flavouring and perfume. Patent DE 4126997.

Moradi, H.; Mohammad, A.A.; Nahvi, I. (2013) Improved γ-decalactone production from Castor Oil by Fed-Batch Cultivation of *Yarrowia lipolytica*. Biocatal. Agric. Biotechnol. 2:64–68.

Naughton, F.C.; Duneczky, F.; Swenson, C.R.; Kroplinski, T.; Cooperman, M.C. (1979) Castor oil. In Encyclopedia of Chemical Technology, Vol. 5, Grayson M. ed., New York: John Wiley & Sons.

Neto, R. S.; Pastore, G. M.; Macedo, G. A. (2004) Biocatalysis and biotransformation producing γ-decalactone. J. Food Sci. C 69(9):677-680.

Nicaud, J.-M.; Belin, J.-M.; Pagot, Y.; Endrizzi-Joran, A. (1996) Bioconversion of substrate with microbe auxotrophic for compound in medium deficient in this compound. FR2734843

Nicaud, J.-M.; Madzac, C.; van den Broek, P.; Gysler, C; Duboc, P.; Niederberger, P.; Gaillardin, C. (2002) Protein expression and secretion in the yeast *Yarrowia lipolytica*. FEMS Yeast Res. 2(3):371-379.

Nielsen, D. R.; Daugulis, A. J.; McLellan, P. J. (2003) A novel method of simulating oxygen mass transfer in two-phase partitioning bioreactors. Biotechnol. Bioeng. 83:735-742.

Nitschke, M.; Pastore, G. M. (2002) Biossurfactantes: propriedades e aplicações. Quim. Nova 25(5):772- 776.

Oh, Y.S.; Maeng, J.; Kim, S.J. (2000) Use of microorganism immobilized polyurethane foams to absorb and degrade oil on water surface. Appl. Microbiol. Biotechnol. 54:418–423.

Ohgaki, S.; Hwang, S.L.; Matsumoto, J. (1978) Kinetics of Biological clogging during artificial recharge attached microbial growth in a continuous stirred tank reactor. Water Res. 12: 243 250.

Okui, S.; Uchiyama, M.; Mizugaki, M. (1963) Metabolism of hydroxy fatty acids: II. Intermediates of the oxidative breakdown of ricinoleic acid by genus *Candida.* J. Biochem. 54:536-540.

Oliveira, R. (1997). Understanding adhesion: a means for preventing fouling. Exp Therm Fluid Sci. 14:316-322.

Olsson, L.; Nielsen, J. (1997) On-line in situ monitoring of biomass in submerged cultivations. Tibtech. 15: 517-522.

Ory, I., Romero, L., Cantero, D. (2004). Optimization of immobilization conditions for vinegar production. Siran, wood chips and polyurethane foam as carriers for *Acetobacter aceti*. Process Biochem. 39: 547–555.

Ory, R.L.; St. Angelo, A.J.; Altschul, A.M. (1960) Castor bean lipase: action on its endogenous substrate. J. Lipid Res. 1:208-213.

Ostle, A.G.; Holt, J.G. (1982) Nile blue A as a fluorescent stain for poly-β-hydroxybutyrate. Appl. Environ. Microbiol. 44:238-241.

Osumi, M.; Fukuzumi, F.; Yamada, N.; Nagatani, T., Teranishi, Y.; Tanaka, A.; Fukui, S. (1975) Surface structure of some *Candida* yeast cells grown on n-alkanes. J. Ferment. Technol. 53:244:248.

Page, G. V.; Eilerman, R. (1996) Process for the preparation of gamma and delta lactones. European Patent EP0417203.

Pagot, Y. (1997) Étude des mécanismes cellulaires et moléculaires de la β-oxydation peroxysomale chez les levures. Effets sur la biotransformation du ricinoléate de méthyle en γ-décalactone. PhD Thesis, Université de Bourgogne, Dijon, France.

Pagot, Y.; Le Clainche, A. ; Nicaud, J.-M. ; Waché, Y. ; Belin, J.-M. (1998) Peroxisomal β-oxidation activities and γ-decalactone production by the yeast *Yarrowia lipolytica*. Appl. Microbiol. Biotechnol. 49:295-300.

Paik, H.D.; Glatz, B.A. (1994) Propionic acid production by immobilized cells of a propionate-tolerant strain of *Propionibacterium acidipropionici*. Appl. Microbiol. Biotechnol.42(1):22-7.

Papaparaskevas, D.; Christakopoulos, P.; Kekos, D.; Macris, B.J. (1992) Optimizing production of extracellular lipase from *Rhodotorula glutinis*. Biotechnol. Lett. 14:397–402.

Papanikolaou, S.; Aggelis, G. (2003) Modeling mipid accumulation and degradation in *Yarrowia lipolytica* cultivated on industrial fats. Curr. Microbiol. 46:398-402.

Pan, P.; Hua, Q. (2012) Reconstruction and *In Silico* Analysis of Metabolic Network for an Oleaginous Yeast, *Yarrowia lipolytica*. Plos One 7(12): e51535.doi:10.1371/journal.pone.0051535.

Park, J.K.; Chang, H.N. (2000) Microencapsulation of microbial cells. Biotechnol. Adv. 18:303–319.

Peter, M.K.; Margaritis, A.; Bergougnou, M.A.; Yu, J.T.; Qin, Y. (2006) Influence of the baffle clearance design on hydrodynamics of a two riser rectangular airlift reactor with inverse internal loop and expanded gas–liquid separator. Chem. Eng. J. 121:17–26.

Picataggio, S.; Rohrer, T.; Deanda, K.; Lanning, D.; Reynolds, R.; Mielenz, J.; Eirich, L.D. (1992) Metabolic engineering of *Candida tropicalis* for the production of long chain dicarboxylic acids. Bio. Tech. 10:894–898.

Pilkington, P.H.; Margaritis, A.; Mensour, N.A.; Russel, I. (1998) Fundamentals of immobilized yeast cells for continuous beer fermentation: a review. J. I. Brewing. 104: 19-31.

Pokorny, D.; Friedrich, J.; Cimerman, A. (1994) Effect of nutritional factors on lipase biosynthesis by *Aspergillus niger*. Biotechnol. Lett. 16:363–366.

Pons, M.N.; Vivier, H.; Dodds, J. (1997) Particle Shape Characterization using Morphological Descriptors. Part. Part. Syst. Charact. 14: 272-277.

Pons, M.N.; Vivier, H. (1998) Beyond filamentous species. Adv. Biochem. Eng. Biotechnol. 60:61.

Prado, G.H.C.; Saldana, M.D.A. (2013) Optimization of Enzymatic Hydrolysis of Sacha Inchi Oil using Conventional and Supercritical Carbon Dioxide Processes. J. Am. Oil Chem. Soc. 90:731-742.

Prathumpai, W.; Flitter, S.J.; Mcintyre, M.; Nielsen, J. (2004) Lipase production by recombinant strains of *Aspergillus niger* expressing a lipase-encoding gene from *Thermomyces lanuginosus*. Appl. Microbiol. Biotechnol. 65:714-719.

Puthli, M.S.; Rathod, V.K.; Pandit, A.B. (2006) Enzymatic hydrolysis of castor oil: Process intensification studies. Biochem. Eng. J. 3:31-41.

Rabenhorst, J.; Gatfield, I. (2000) Process for the production of γ-decalactone. European

Patent 0997533.

Rabenhorst, J.; Gatfield, I. L. (2001) Method of producing γ-decalactone. Patent WO 0024920.

Rabenhorst, J.; Gatfield, I. L. (2002) Method of producing γ-decalactone using *Yarrowia lipolytica* strain HR 145 (DSM 12397). US patent 6451565.

Ramakrishna, S.V.; Prakasham, R.S.(1999) Microbial fermentations with immobilized cells. Curr .Sci.77: 87-100.

Rapp, R.; Backhaus, S. (1992) Formation of extracellular lipases by filamentous fungi, yeasts, and bacteria. Enzyme Microb. Technol. 14:938–943.

Rathod, V.K.; Pandit, A.B. (2009) Effect of various additives on enzymatic hydrolysis of castor oil. Biochem. Eng. J. 47:93–99.

Ravaine, V.; Bibette, J.; Henry, N. (2002) Wetting of liquid droplets on living cells. J. Colloid Interface Sci. 255: 270-273.

Reis, N.; Gonçalves, C.; Aguedo, M.; Gomes, N.; Teixeira, J.A.; Vicente, A.A. (2006) Application of a novel oscillatory flow micro-bioreactor to the production of γ-decalactone in a two immiscible liquid phase medium. Biotechnol. Lett. 28:485-490.

Rochex, A.; Lecouturier, D. ; Pezron, I. ; Lebeault, J.M. (2004) Adhesion of a *Pseudomonas putida* strain isolated from a paper machine to cellulose fibres. Appl. Microbiol. Biotechnol. 65: 727–733.

Rols, J.L.; Goma, G. (1989) Enhancement of oxygen transfer rates in fermentation using oxygen-vectors. Biotechnol. Adv. 7:1 – 8.

Royer, A.; Naulet, N.; Mabon, F.; Lees, M.; Martin, G.J. (1999a) Stable isotope characterization of olive oils. II. Deuterium distribution in fatty acids studied by nuclear magnetic

resonance. J. Am. Oil Chem. Soc. 76:365-373.

Royer, A.; Gerard, C.; Naulet, N.; Lees, M.; Martin, G.J. (1999b) Stable isotope characterization of olive oils. I. Compositional and carbon-13 profiles of fatty acids. J. Am. Oil Chem. Soc. 76:357-363.

Russ, C.R. (1995) The Image Processing Handbook, CRC Press, Boca Raton.

Rymowicz, W.; Rywinska, A.; Marcinkiewicz, M. (2009) High yield production of erythriol from raw glycerol in fed-batch cultures of *Yarrowia lipolytica*. Biotechnol. Lett. 31(3):377-380.

Saarela, U.; Leiviska, K.; Juuso, E. (2003) Modelling of a fed-batch fermentation process. Report A No. 21, June 2003. Control Engineering Laboratory, University of Oulu Finland.

Salleh, A.B.; Musani, R.; Basri, M.; Ampon, K.; Yunus, W.M.Z.; Razak, C.N.A. (1993) Extra- and intra-cellular lipases from a thermophilic *Rhizopus oryzae* and factors affecting their production. Can. J. Microbiol. 39:978–981.

Samuel, T.; Cockx, A.; Line, A. (2005) Global modeling of a gas–liquid–solid airlift reactor. Chem. Eng. Scien. 60:5991-6003.

Saswathi, N.; Sheela, R.; Jamuna, R.; Ramakrishna, S.V. (1995) Synthesis of cyclodextrin glycosyl transferase by immobilized cells of *Bacillus circulans*. Bioproc. Eng. 12:283–6.

Saudi, M.S.; Krishnan, J.; Ling, T.H.; Veluri, M.V.P.S. (2013) Enhancement of oxygen mass transfer and gas holdup using palm oil in stirred tank bioreactors with xanthan solutions as simulated viscous fermentation broths. Biomed. Res. Int. Article ID 409675.

Schrader, J.; Etschmann, M. M. W.; Sell, D.; Hilmer, J.-M.; Rabenhorst, J. (2004) Applied biocatalysis for the synthesis of natural flavour compounds – current industrial processes and future prospects. Biotechnol. Lett. 26:463-472.

Serrano-Carréon, L.; Flores, C.; Galindo, H. (1997) γ-Decalactone production by

Trichoderma harzianum in stirred bioreactors. Biotechnol. Prog. 13:205-208.

Sharon, C.; Furugoh, S.; Yamakido, T.; Ogawa, H. I.; Kato, Y. (1998) Purification and characterization of a lipase from *Pseudomonas aeruginosa* KKA-5 and its role in castor oil hydrolysis. J. Ind. Microbiol. Biotechnol. 20: 304-307.

Shimada, Y.; Sugihara, A.; Nagao, T.; Tominaga, Y. (1992) Induction of *Geotrichum candidum* lipase by long-chain fatty acids. J. Ferment. Bioeng. 74:77–80.

Siek, T. J.; Albin, I. A.; Sather, L. A.; Lindsay, R. C. (1971) Comparison of flavor thresholds of aliphatic lactones with those of fatty acids, esters, aldehyde, alcohols and ketones. J. Dairy Sci. 54:1-9.

Smucker, R.A.; Cooney, J.J. (1981) Cytological responses of *Cladosporium resinae* when shifted from glucose to hydrocarbon medium. Can. J. Microbiol. 27: 1209-1218.

Souchon, I. (1994) Extraction en continu de lactones produites par voie microbiologique. Thése de Doctorat de l'Université de Bourgogne - ENSBANA, Dijon, France.

Souchon, I.; Spinnler, H.E.; Dufossé, L.; Voilley, A. (1998) Trapping of γ-decalactone by adsorption on hydrophobic sorbents: application to the bioconversion of methyl ricinoleate by the yeast *Sporidiobolus salmonicolor*. Biotechnol. Tech. 12:109–113.

Spinnler, H. E.; Giniès, C.; Khan, J. A.; Vulfson, E. N. (1996) Analysis of metabolic pathways by the growth of cells in the presence of organic solvents. Proc. Natl. Acad. Sci. USA 93:3373-3376.

Strehaiano, P.; Ramon-Portugal, F.; Taillandier, P. (2006) Yeasts as biocatalysts. *In* Yeasts in Food and Beverages ed. Querol, A. and Fleet, G.H. Heidelberg: Springer.

Suresh, S.; Srivastava, V.C.; Mishra, I.M. (2009) Techniques for oxygen transfer measurement in bioreactores: a review. J. Chem. Technol. Biotechnol. 84: 1091-1103.

Szabo, R. (2001) Cla4 protein kinase is essential for filament formation and invasive growth of *Yarrowia lipolytica*. Molec. Genet. Genom. 265:172-179.

Szaniszlo, P.J. (1985) Fungal dimorphism with emphasis on fungi pathogenic for humans. Plenum, New York.

Taguchi, H.; Humphrey, A. (1966) Dynamic method of the volumetric oxygen transfer coefficient in the fermentation systems. J. Ferm. Technol. 44:881-889.

Tahara, S.; Fujiwara, K.; Mizutani, J. (1973) Neutral constituents of volatiles in cultured broth of *Sporobolomyces odorus*. Agric. Biol. Chem. 37: 2855.

Titorenko, V.I.; Ogrydziak, D.M.; Rachubinski, R.A. (1997) Four distinct secretory pathways serve protein secretion, cell surface growth, and peroxisome biogenesis in the yeast *Yarrowia lipolytica*. Mol. Cell. Biol. 17:5210–5226.

Titorenko, V.I.; Smith, J.J.; Szilard, R.K.; Rachubinski, R.A. (2000) Peroxisome biogenesis in the yeast *Yarrowia lipolytica*. Cell Biochem. Biophys. 32: 21–26.

Trelles, J.A.; Quiroga, F.; Britos, C.; Smolko, E.E.; Grasselli, M. (2010). Immobilization of bacteria in microgel grafted onto macroporous polyethylene. Radiat Phys. Chem. 79:241.

Tribe, A.; Briens, C.L.; Margaritis, A. (1995) Determination of the volumetric mass transfer coefficient (k_La) using the dynamic "gas out-gas in" method: analysis of errors caused by dissolved oxygen probes. Biotechnol. Bioeng. 46:388–392.

Turki, S.; Ayed, A.; Chalghoumi, N.; Weekers, F.; Thonard, P.; Kallel, H (2010) An enhanced process for the production of a highly purified extracellular lipase in the non-conventional yeast *Yarrowia lipolytica*. Appl. Biochem. Biotechnol. 160:1371-1385.

van der Walt, J.P.; von Arx, J.A. (1980) The yeast genus *Yarrowia* gen. nov. A. Van Leeuw. 46:517–521

Vandamme, E.J.; Soetaert, W. (2002) Bioflavours and fragrances via fermentation and biocatalysis. J. Chem. Technol. Biotechnol. 77:1323-1332.

Vanden Bossche, A.; Odds, F.C.; Kerridge, D. (1993) Dimorphic fungi in biology and medicine. Plenum, New York.

Verbelen, P.J., Schutter, D.P., Delvaux, F., Verstrepen, K.J., and Delvaux, F.R. (2006). Immobilized yeast cell systems for continuous fermentation applications. Biotechnol. Lett. 28: 1515-1525.

Villeneuve, P.; Muderhwa, J.M.; Graille, J.; Haas, M.J. (2000) Customizing lipases for biocatalysis: a survey of chemical, physical and molecular biological approaches. J. Mol. Catal. B: Enzym. 4:113-148.

Voet, D.; Voet, J.G. (1990) Biochemistry. John Wiley & Sons, New York.

Waché, Y.; Pagot, Y.; Nicaud, J.-M.; Belin, J.-M. (1998) Acyl-CoA oxidase, a key step for lactone production by *Yarrowia lipolytica*. J. Mol. Catal. B: Enzymatic 5:165-169.

Waché, Y.; Bergmark, K.; Courthaudon, J.-L.; Aguedo, M.; Nicaud, J.-M.; Belin, J.-M. (2000a) Medium-size droplets of methyl ricinoleate are reduced by cell-surface activity in the γ-decalactone production by *Yarrowia lipolytica*. Lett. Appl. Microbiol. 30: 183-187.

Waché, Y.; Laroche, C.; Bergmark, K.; Moller-Andersen, C. ; Aguedo, M.; Le Dall, M.T.; Wang, H.; Nicaud, J.M.; Belin, J.M. (2000b) Involvement of acyl-CoA oxidase isozymes in biotransformation of methyl ricinoleate into γ-decalactone by *Yarrowia lipolytica*. Appl. Environ. Microbiol. 66:1233-1236.

Waché, Y.; Aguedo, M.; Choquet, A.; Gatfield, I.; Nicaud, J.M.; Belin, J.M. (2001) Role of β-oxidation enzymes in γ-decalactone production by the yeast *Yarrowia lipolytica*. Appl. Environ. Microbiol. 67(12):5700–5704.

Waché, Y.; Aguedo, M.;Ledall, M.T.; Nicaud, J.M.; Bellin, J.M. (2002). Optimization of *Yarrowia lipolytica*'s β-oxidation pathway for γ-decalactone production. J. Mol. Catal. B: Enzym. 19-20, 347-351.

Waché, Y.; Aguedo, M.; Nicaud, J.-M.; Belin, J.-M. (2003) Catabolism of hydroxyacids and biotechnological production of lactones by *Yarrowia lipolytica*. Appl. Microbiol. Biotechnol. 61:393-404.

Wang, H.; Le Dall, M.-T.; Waché, Y.; Laroche, C.; Belin, J.-M.; Gaillardin, C.; Nicaud, J.-M. (1999) Evaluation of acyl coenzyme A oxidase (Aox) isozyme function in the n-alkane-assimilating yeast *Yarrowia lipolytica*. J. Bacteriol. 181:5140.

Wang, X.-D.; Mauvais, G.; Cachon, R.; Diviés, C.; Feron, G. (2000) Addition of reducing agent dithiothreitol improves 4-decanolide synthesis by the genus *Sporidiobolus*. J. Biosci. Bioeng. 90: 338 – 340.

Wang, Y.; Tian, Y.; Han, B.; Zhao, H.; Bi, J.; cai, B. (2007) Biodegradation of phenol by free and immobilized *Acinetobacter sp.*strain PD12. J. Environ. Sci. 19: 222-225.

Walker, GM. (1998) Yeast Physiology and Biotechnology. John Wiley & Sons.

Wickerman, L.J.; Kurtzman, C.P.; Herman, A.I. (1970) Sexual reproduction in *Candida lipolytica*. Science. 167:1141-1147.

Wise, W. (1951) The measurement of the aeration of culture media. J. Gen. Microbiol. 5:167-177.

Wolf, K. (1996) Non-Conventional Yeast in Biotechnology. A Handbook. Springer, Berlin.

Wu, L.; Ge, G.; Wan, J. (2009) Biodegradation of oil wastewater by free and immobilized *Yarrowia lipolytica* W29. J. Environ. Sci. 21:237-242.

Yamane, T.; Shimizu, S. (1984) Fed-batch techniques in microbial processes. Adv. Biochem,

Eng. Biotechnol. 30:147-194.

Yang, J.D.; Wang, N.S. (1992) Oxygen mass transfer enhancement via fermentor headspace pressurization. Biotechnol. Prog. 8:244-251.

Yarrow, D. (1972) Four new combinations in yeasts. A Van Leeuw 38:357-360.

Yoshida, F.; Yamane, T.; Miyamoto, M. (1970) The increase of oxygen diffusion rates in broths adding organic substrates. Ind. Eng. Chem. Proc. Des. Dev. 9:570-577.

Yu, J. ; Yue, G. ; Zhong, J. ; Zhang, X. ; Tan, T. (2010) Immobilization of *Saccharomyces cerevisiae* to modified bagasse for ethanol production. Renew. Energ. 35:1130–1134.

Zacheus, O.M.; Livanainen, E.K.; Nissinen, T.K.; Lehtola, M.J.; Martikainen, P.J. (2000) Bacterial biofilm formation on polyvinyl chloride, polyethylene and stainless steel exposed to ozonated water. Water Res. 34:63-70.

Zgura, I.; Beica, T.; Mitrofan, I.L.; Mateias, C.G.; Pirvu, D.; Patrascu, I. (2010) Assessment of the impression materials by investigation of the hydrophilicity Dig. J. Nanomater. Bios. 5:749–755.

Zhang, T.; Wang, T.; Wang, J. (2006) Analysis and Measurement of Mass Transfer in Airlift Loop Reactors. Chinese J. Chem. Eng. 14:604-610.

Zhang, H. (2007) Evaluation of practical process aspects for Lipozyme TL IM catalyzed bulk fat modification in a batch reactor. Open Biotechnol. J. 1:72-80.

Zhonghuo, D.; Tiefeng, W.; Zhang, N.; Wang, Z. (2010) Gas holdup, bubble behavior and mass transfer in a 5m high internal-loop airlift reactor with non-Newtonian fluid. Chem. Eng. J. 160: 729-737.

Zinjarde, S.S.; Pant, A. (2000) Crude oil degradation by free and immobilized cells of *Yarrowia lipolytica* NCIM 3589. J. Environ. Sci. Saúde A Tox. Hazard Subst. Environ. Eng. 35 :755-763.